Dissertation

The role of bone morphogenetic protein signaling in the induction and maintenance of peripheral tolerance

submitted by

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Statutory Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all those individuals and organizations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the “Guidelines of the Medical University of Graz on Good Scientific Practice”.

Tommaso Sconocchia
Graz, November 2020.

Disclosures

Parts of this thesis have been published in the following research article (Sconocchia *et al.*, 2020):

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Tommaso Sconocchia designed and performed most of the experiments, analyzed the data, and wrote the manuscript.

Mathias Hochgerner, Thomas Bauer, and Maria Sibilia provided data and samples form the mouse experiments (Institute of Cancer Research, Department of Internal Medicine I, Medical University of Vienna, Austria).

Theresa Benezeder, Peter Wolf (Department of Dermatology, Medical University of Graz, Austria), and Clemens Painsi (Department of Dermatology, State Hospital Klagenfurt, Austria) provided skin samples from psoriasis patients and healthy individuals.

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Herbert Strobl (Otto Loewi Research Center, Division of Immunology and Pathophysiology, Medical University of Graz, Austria) secured funding, supervised the project and co-wrote the manuscript.

All co-authors contributed to data analysis and manuscript editing.

All co-authors declare that they have no conflicts of interest to disclose and have agreed to use their data in the thesis.

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Abbreviations and Definitions

APC	Antigen presenting cell
BG	Birbeck granule
BMP	Bone morphogenetic protein
BMPR1a	BMP type Ia receptor
cDC	Conventional dendritic cell
CFSE	carboxyfluorescein siccinimidyl ester
DC	Dendritic cell
dDC	Dermal dendritic cell
DM	Dorsomorhpin
EpCAM	Epithelial cell adhesion molecule
FLT3L	FMS-related receptor tyrosine kinase 3 ligand
GM-CSF	Human granulocyte-macrophage colony stimulating factor
HPC	Hematopoietic progenitor cell
IDEC	Inflammatory dendritic epidermal cell
IFNγ	Interferon gamma
IL-17	Interleukin-17
IL-2	Interleukin-2
IL-22	Interleukin-22
IL-4	Interleukin-4
iTreg	Induced regulatory T cell
KO	Knockout
LC	Langerhans cell
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MoDC	Monocyte-derived dendritic cell
nTreg	Natural thymus-derived regulatory T cell
pDC	Plasmacytoid dendritic cell
SB	SB431542
SCF	Stem cell factor
slan-DCs	6-Sulfo LacNAc DCs

Tfh	Follicular T helper cells
TGF-β1	Transforming growth factor-β1
TGFβR1	TGFβ type I receptor
Th	T helper
TNFα	Tumor necrosis factor-a
TPO	Thrombopoietin
Tr1	Inducible T regulatory type 1 cell
Treg	Regulatory T cell
WT	Wild-type

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Zusammenfassung

Dendritische Zellen (DCs) sind professionelle antigenpräsentierende Zellen (APCs) und ein Bindeglied zwischen dem angeborenen und adaptiven Immunsystem. DCs spielen eine wichtige Rolle, da sie Antigene aufnehmen, verarbeiten und anschließend den T-Zellen präsentieren, wodurch eine koordinierte Immunantwort gegen Pathogene ermöglicht wird. DCs können in vielen Organen und Geweben, sowie auch in der Haut, gefunden werden. Hierbei weist die Epidermis eine spezifische Gruppe von DCs auf, welche als Langerhans Zellen (LCs) bezeichnet werden. LCs bilden ein dichtes zelluläres Netzwerk aus und vermitteln unter physiologischen Bedingungen Immuntoleranz durch die Vermehrung regulatorischer T-Zellen (Treg Zellen). Durch epidermale Faktoren können LCs manipuliert bzw. deren Differenzierung beeinflusst werden. Vor kurzem wurde beschrieben, dass das Knochenmorphogenetische Protein BMP-7 (englisch bone morphogenetic protein, BMP), welches mit dem TGF-ß-Signalweg assoziiert ist, in der psoriatischen Epidermis abberant exprimiert wird. Diese abweichende Expression kann zur Differenzierung von Monozyten in LCs mit entzündungsassoziierten LC-ähnlichen DC-Charakteristika führen. Deren funktionelle Charakteristika sind jedoch noch nicht vollständig analysiert. Auch die Rolle von Treg Zellen in Psoriasis hat zunehmend an Bedeutung gewonnen. Tatsächlich wurde in Mausstudien gezeigt, dass sich Treg Zellen in Psoriasis-ähnlichen Hautläsionen akkumulieren und den Schweregrad der Erkrankung limitieren können.

Angesichts der Tatsache, dass LCs bei der Vermehrung von Treg Zellen in der Haut eine entscheidende Rolle spielen, der Vorgang, welcher zur Akkumulierung der Treg Zellen während der Entzündung in der Haut führt jedoch noch nicht geklärt ist, haben wir die Rolle von BMP7 und seinem Rezeptor BMPR1a in diesem Prozess untersucht.

In dieser Arbeit beschreiben wir einen Zusammenhang zwischen dem BMP7/BMPR1a Signalweg, LCs/DCs und der Akkumulation von Treg Zellen in Psoriasis. Immunhistologische Untersuchungen von Psoriasis Patienten und gesunden Personen der Kontrollgruppe sowie auch die Versuche mit dem Psoriasis-imitierenden Modellsystems mit Mäusen, welche keinen BMPR1a besitzen, zeigten eine positive Korrelation zwischen der BMP7 Expressionsintensität in der Haut und der Akkumulation von Treg Zellen in psoriatischen Läsionen. Zusätzlich zeigte ein Teil der $FoxP3^+$ Zellen in psoriatischen Läsionen aktive BMP-Signalwirkung. Darüber hinaus konnten wir zeigen, dass die BMPR1a-Signalwirkung in entzündungsassoziierten LCs und DCs mit einer reduzierten Entzündung in der Haut einhergeht. Weiters fördern sie die Differenzierung von naiven $CD4^+$ T-Zellen zu Treg Zellen, in einem Prozess, welcher von einer BMPR-vermittelten CD25/IL-2 Induktion abhängig ist.

Aufgrund unserer Daten sind wir zu dem Schluss gekommen, dass der BMP-Signalweg in Psoriasis eine regulatorische Rolle spielt, indem er die Entzündung eindämmt. Wir sind davon überzeugt, dass BMP7 und nachgeschaltete Signalwege in Psoriasis stärker exprimiert sind und innerhalb der Läsionen eine positive Assoziation mit Treg Zellen aufweisen. BMPR1a steigert die Treg-stimulierenden Fähigkeiten von entzündungsassoziierten LCs/DCs; zusätzlich kann auch lokal sezerniertes BMP die Treg Differenzierung direkt fördern.

Abstract

Dendritic cells (DCs) are professional antigen presenting cells (APCs) that link the innate and adaptive immunity. DCs play a strong role in immunity by taking up, processing, and presenting antigens to T cells in order to coordinate an immune response against pathogens. DCs can be found in many organs and tissues and are also present in the skin. The epidermis contains a unique DC subset known as Langerhans cell (LC). LCs form a dense cellular network in the epidermis and under steady-state conditions maintain immune tolerance in the skin by expanding tissue resident regulatory T (Treg) cells. LCs are susceptible to programming by factors present in the epidermis. Recently, a member of the TGF-β family known as bone morphogenetic protein (BMP)7 was described to be aberrantly expressed in the epidermis during psoriasis and to differentiate moncytic cells into LCs with characteristics of inflammation-associated LC-like DCs. However, their functional characteristics are still not fully understood. The role of Treg cells during psoriasis has recently gained more importance. In fact, murine studies described that Tregs accumulate in psoriasis-like skin lesions and have a role in limiting the severity of the disease.

Given the role of LCs in expanding Tregs in the skin and that the mechanisms mediating Treg accumulation during skin inflammation remain elusive, we investigated within this thesis the role of BMP7 and its receptor BMPR1a in Treg accumulation during skin inflammation.

We here described a link between BMP7/BMPR1a signaling, LCs/DCs and Treg accumulation during psoriasis. Immunohistology of psoriasis patients and healthy controls together with the analysis of a psoriasis-like inflammation model in mice lacking BMPR1a described a positive correlation between BMP7 expression intensity in the skin and Treg accumulation in psoriatic lesions. In addition, a portion of $FoxP3^+$ cells in psoriatic lesions exhibited active BMP signaling. Moreover, we described that BMPR1a signaling in inflammation-associated LCs and DCs is associated with lower skin inflammation and that it increases the capacity of LCs/DCs to promote Treg differentiation from naïve $CD4^+$ T cells in a mechanism that is dependent on BMPR-mediated CD25/IL-2 induction.

We concluded that BMP signaling plays a regulatory role during psoriasis by limiting inflammation. We suggest a model in which BMP7 and its downstream signaling pathway are strongly expressed in psoriasis and positively associate with Tregs within the lesions. BMPR1a promotes inflammation-associated LCs/DCs to gain enhance Treg stimulatory abilities and locally secreted BMPs can also promote Treg differentiation in a direct manner.

1. Introduction

1.1. Dendritic cells

Dendritic cells (DCs) are a heterogeneous population of immune cells that are defined as professional antigen presenting cells (APCs) (Haniffa, Gunawan and Jardine, 2015). Their main role consists in the uptake of antigens, followed by migration to the draining lymph-nodes, and subsequent presentation of the antigen to T cells with the aim of initiating the immune response, thus protecting the host from infectious pathogens (Banchereau and Steinman, 1998). DCs interact with and stimulate naïve $CD4^+$ T cells by presenting internalized and processed extracellular antigens through the major histocompatibility complex (MHC) class II, and based on the cytokines that are secreted, they polarize T cells towards different T helper (Th) subsets (Banchereau *et al.*, 2000). In addition, DCs are the most effective cell type that can cross-present antigens. Cross-presentation of antigens consists in the internalization of extracellular antigens, followed by their presentation through the MHC class I to stimulate naïve $CD8^+$ T cells into cytotoxic $CD8^+$ T cells (Klechevsky *et al.*, 2008). DC subsets can be classified based on different criteria including their origin (myeloid or lymphoid), surface marker expression, and location (blood circulating, tissue resident, lymph-node resident) (Zaba, Krueger and Lowes, 2009).

1.1.1. Human DC subsets

Due to their heterogeneity, numerous dendritic cells subsets have been identified. However, due to their complexity, it is not fully understood how many different subsets exist, and what is their specific function. The traditional classification of dendritic cells subsets relied mainly on the expression of certain markers, morphological features, and the developmental origin of the cells. They were classified into the following groups: $CD11c^+$ conventional DCs (cDCs) which can be further divided into $CD141^+$ cDC1 and $CD1c^+$ cDC2, and $CD11c^{low}CD123^+$ plasmacytoid DCs (pDCs). By using an unbiased approach by means of single cell RNA sequencing technology Villani *et al*. could redefine the classification of different DC subsets leading to the identification of new DC subsets. The new classification today includes: $CD141^+CLEC9A^+IRF8^+$ cDC1, $SIRP\text{-}\alpha^+CD1c^+IRF4^+$ DCs (encompassing $BTLA^+CD5^+$ cDC2 and $CD163^+$ DC3s), $CD1c^-CD141^-$ DC4, $AXL^+SIGLEC6^+$ DC5 (AS DCs), and DC6 (also known as $CD123^+$ pDCs) (Villani *et al.*, 2017). A certain layer of complexity arises from AS DCs, which were suggested to be either their own lineage or to act as a precursor for cDCs (See *et al.*, 2017; Villani *et al.*, 2017). cDC1s and cDC2s derive from a

common dendritic cell precursor (CDP) in a FLT3L-dependent mechanism (Lee *et al.*, 2015). DC3s instead develop independently from a DC-committed (CDP) or monocyte-committed (cMoP) by a process stimulated by GM-CSF *in vitro* and *in vivo* in humanized mice (Bourdely *et al.*, 2020).

1.1.2. Human skin resident DC subsets

In healthy human skin two main DC subsets are present. These have been identified as Langerhans cells (LCs), and dermal DCs (dDCs). dDCs are of myeloid origin and can be further divided into: $CD1c^{+}CD1a^{+}$ dDCs, $CD141^{hi}$ dDCs and $CD14^{+}$ dDCs (Lenz *et al.*, 1993; Haniffa *et al.*, 2012) (Figure 1, left side). During skin inflammation, a third heterogeneous group of “inflammatory” DCs is present (Figure 1, right side). A fourth group, known as plasmacytoid DCs (pDCs) are also present in the human skin and have been mostly detected in inflamed skin (Wenzel and Tüting, 2008; Gregorio *et al.*, 2010). Their presence in healthy skin is debated with studies detecting very low numbers of pDCs in healthy skin (Bangert *et al.*, 2003; Ebner *et al.*, 2004).

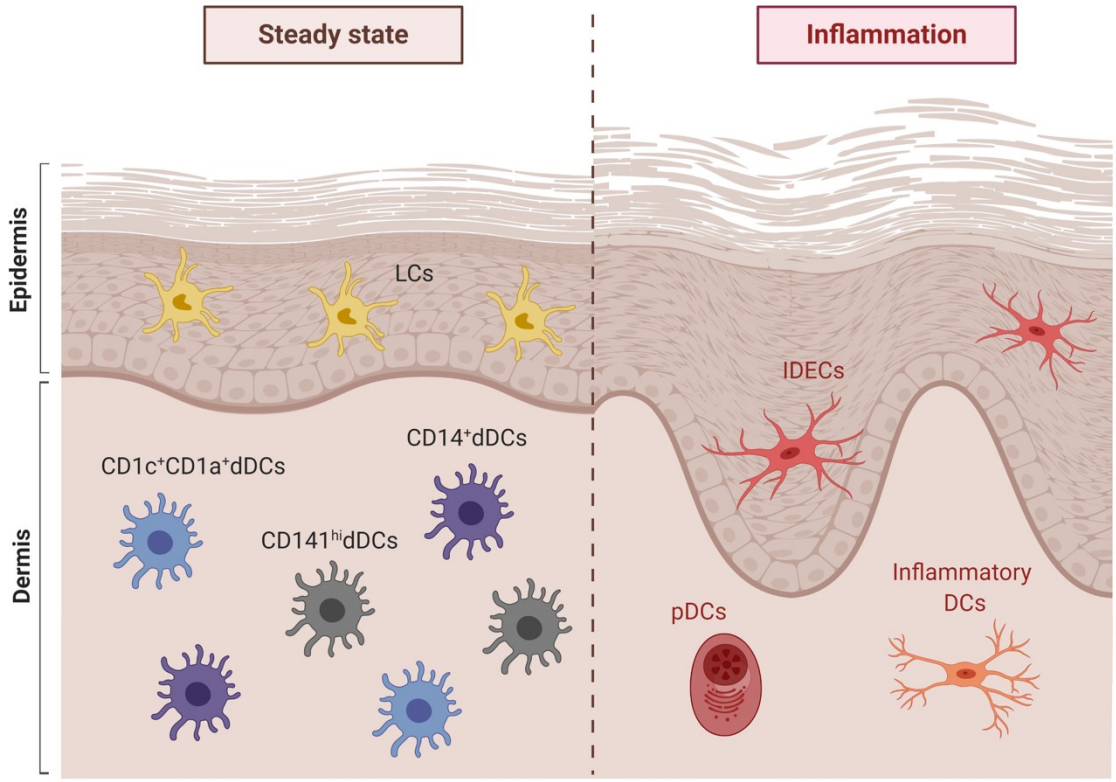

Figure 1. Skin dendritic cell subsets. In healthy human skin, Langerhans cells form a dense cellular network in the epidermis. In the dermal compartment, two main dermal dendritic cell groups can be identified based on their expression of CD1a and CD14. During inflammation, the skin is populated by “inflammatory” dendritic cells (also includes IDECs) and plasmacytoid dendritic cells. This figure was designed with BioRender.com.

Langerhans cells

Paul Langerhans first described LCs in 1868 while he was a medical student (Langerhans, 1868). Due to their dendritic shape resembling neurons, they were first thought to be part of the nervous system. With Ralph Steinmann, it was later discovered that in fact LCs are capable of antigen presentation and were then classified as part of the DC family (Schuler and Steinman, 1985). Today, it is known that LCs have an embryonic origin and are mainly maintained through self-renewal, leading to a debate regarding their classification. When their developmental origin is used for their classification, then LCs are to be inserted in

the macrophage lineage. However, functionally LCs resemble DCs (Doebel, Voisin and Nagao, 2017). Recently, elegant studies have attributed a dual identity of LCs as they express Zbtb46 (a transcription factor which enforces DC identity), but derive from Mafb-expressing cells, signifying macrophage origin (Wu *et al.*, 2016).

LCs populate namely the epidermis in close contact with keratinocytes where they form a dense cellular network. They comprise 3-5% of the nucleated cells in the epidermis and reside in the suprabasal keratinocyte layer. LCs obtain a DC phenotype by expressing MHC class II and CD1a, the latter being structurally related to MHC proteins and involved in lipid antigen presentation (Barral and Brenner, 2007). Other important LC markers include the epithelial cell adhesion molecule (EpCAM), E-Cadherin/CD324, and the C-type lectin Langerin/CD207 (Valladeau *et al.*, 2000). EpCAM and E-Cadherin are involved in regulating the adhesion of LCs to keratinocytes (Tang *et al.*, 1993; Gaiser *et al.*, 2012). Langerin is involved in the formation of Birbeck granules (BG). These structures are racket-shaped endosomal organelles which are characteristic to LCs (Mc Dermott *et al.*, 2002).

LCs act as immune sentinels, they can extend their dendrites towards the *stratum corneum* whilst maintaining barrier integrity and can probe for the presence of pathogens (Kubo *et al.*, 2009). Once a pathogen is encountered, LCs become activated by upregulating MHC class II and costimulatory molecules, and by downregulating molecules like E-Cadherin, which allows them to detach from keratinocytes and migrate to the skin-draining lymph nodes. In the lymph nodes, LCs interact and prime T cells, leading to an immune response against the pathogen (Klechevsky *et al.*, 2008). In the steady-state, LCs are also involved in the maintenance of skin homeostasis. This is largely carried out by their ability to expand regulatory T cells (Tregs) (Seneschal *et al.*, 2012; Kitashima *et al.*, 2018). Regarding this ability, it is not fully clear whether this happens in the skin, lymph-nodes or both. Interestingly, recent studies have shown that in the steady-state LCs and skin resident Tregs are in close proximity at the epidermal-dermal junction, and that LCs isolated from the skin selectively expand autologous skin resident Tregs *in vitro* (Seneschal *et al.*, 2012).

Dermal dendritic cells

DCs in the human dermis were identified following a study that performed an immunostaining for Factor XIIIa, which identified dendritic shaped cells in the dermis (Cerio *et al.*, 1989). These DCs are known today as dermal DCs (dDCs). These dDCs, which are distinct from epidermal LCs, can spontaneously migrate from *ex vivo* skin explants (Lenz *et al.*, 1993). Analysis of these migrated DCs revealed three different subsets based on their expression of CD1c, CD1a, CD141, and CD14 (Nestle *et al.*, 1993; Morelli *et al.*, 2005; Haniffa *et al.*, 2012)

(Figure 1). These subsets include: CD1c$^+$CD1a$^+$dDCs, CD141^{hi}dDCs, and CD14$^+$dDCs. The largest of these subpopulations are the CD1c$^+$CD1a$^+$dDCs. This subset of dDCs are strong stimulators of naïve CD4$^+$ T cells and can promote both Th1 and Th2 cells (Nestle *et al.*, 1993). They also retain the ability to cross-present antigens to CD8$^+$ T cells. The CD141^{hi}dDCs represent the most recent described subset of DCs in the human dermis. This subset is characterized by high expression of CD141 in combination with no expression of CD14 and low expression of CD1c. They are strong in antigen cross-presentation (Haniffa *et al.*, 2012). The CD14$^+$dDCs represent the smallest of the identified subsets. Around this population, there is some debate because more recent transcriptional analysis has revealed that they are more associated with monocytes/macrophages rather than DCs (Zaba *et al.*, 2007). Although being transcriptionally similar to monocytes/macrophages, this subset retains functional features of DCs like the ability to migrate to the lymph-nodes (Haniffa *et al.*, 2009). CD14$^+$ dDCs are not great stimulators of naïve T cells, but are involved in follicular Th cell differentiation (Klechevsky *et al.*, 2008). In addition, CD14$^+$ dDCs can strongly induce memory T cell activation (Klechevsky *et al.*, 2008).

Inflammatory dendritic cells

During inflammatory conditions, newly differentiated DCs, which are normally not present in steady-state conditions, populate the skin. These DCs represent a heterogeneous population and can be grouped under the term “inflammatory DCs”. The origin of these cells is still not fully clear and different studies are tackling to unveil their origin. Deciphering the true origin of inflammatory DCs, especially in humans, remains a hard task. Mouse studies have shown that these cells derive from Ly6C^{hi} monocytes (Tamoutounour *et al.*, 2013). In humans, transcriptional profiling revealed that inflammatory DCs share resemblance with *in vitro* monocyte-derived dendritic cells (moDCs) (Segura *et al.*, 2013), which are generated by culturing CD14$^+$ monocytes with GM-CSF and IL-4 (Romani *et al.*, 1994). These cells play an essential role in the pathogenesis and evolution of skin diseases like psoriasis and atopic dermatitis.

Given the heterogeneity of this subset of dendritic cells, it is no surprise that these cells are also functionally different. Inflammatory DCs that were shown to produce TNFα and iNOS have been labelled as Tip-DCs (Serbina *et al.*, 2003). Other inflammatory DCs described include also 6-Sulfo LacNAc DCs (slan-DCs) (Ingwersen *et al.*, 2011). These two subsets have been described in psoriatic skin. In atopic dermatitis, pDCs and inflammatory dendritic epidermal cells (IDEC) have been described. IDECs are CD1a$^+$CD206$^+$FCεRI$^+$ myeloid DCs. These cells reside in the epidermis and are distinct from Langerhans cells by their lack of BGs

and lower expression of CD1a (Wollenberg *et al.*, 1996, 2002).

1.2. $CD4^+$ helper T cell subsets

$CD4^+$ T cells, together with $CD8^+$ T cells make up the majority of T lymphocytes in humans. Like other T cells, $CD4^+$ T cells develop in the thymus (Takahama, 2006). Once formed, they find themselves in a "blank" state and are known as naïve $CD4^+$ T cells. This naïve phenotype is characterized by the high expression of CD45RA (De Rosa *et al.*, 2001). Subsequently, they migrate to the secondary lymphoid organs where antigens can be presented to them via MHC class II from APCs (Masopust and Schenkel, 2013). Once an antigen is presented, T cells receive a stimulatory signal which leads to their activation, with downregulation of CD45RA and upregulation of CD45RO (Early and Reen, 1999). During this phase, the cells undergo a strong proliferation and produce different growth factors and cytokines. Based on the cytokines produced, $CD4^+$ T cells can differentiate into different effector subsets (Luckheeram *et al.*, 2012). These are known as T helper (Th) cells and the main subsets described today are Th1, Th2, Th17, Th22, Th9, and follicular T helper cells (Tfh) (described more in detail under the section pro-inflammatory $CD4^+$ T cells), Tregs, and Tr1 cells (described more in detail under the section regulatory $CD4^+$ T cells). $CD4^+$ T cells were coined as "helper" because their main course of action is to secrete cytokines which regulate and assist the immune response (Table 1).

Table 1: CD4$^+$ T helper subsets

Th subset	Differentiation cytokines	Transcription Factors	Effector Cytokines	Function
Th1	IL-12, IFNγ	T-bet, STAT4	IFNγ, IL-2	Intracellular pathogens
Th2	IL-4, IL-2	GATA-3, STAT5	IL-4, IL-5, IL-13, IL-10	Extracellular pathogens
Th17	TGFβ, IL-6, IL-21, IL-23	RORγt, STAT3	IL-17A, IL-17F, IL-21, IL-22	Extracellular bacteria, fungi
Th22	IL-6, TNFα	AhR, T-bet	IL-22	Bacteria, wound healing and barrier integrity
Th9	TGFβ, IL-4	PU.1, IRF4	IL-9	Helminthes
Tfh	IL-6, IL-21	Bcl6, STAT3	IFNγ, IL4, IL-10	B-cell immunity
iTreg	TGFβ, IL-2	FoxP3, STAT5	IL-10, IL-35, TGFβ	Immunosuppression
Tr1	IL-10, IL-27	GATA-3	IL-10, TGFβ, IL-5^{low}, IL-2^{low}, IFNγ^{low}	Immunosuppression

1.2.1 Pro-inflammatory CD4$^+$ Th cells

The subsets described in this section mainly have the function to promote the clearance of intracellular and extracellular pathogens, like viruses, bacteria and fungi. However, a dysregulated activation of these cells can lead to autoimmunity. Th1 and Th2 subsets were

the first to be described (Mosmann *et al.*, 1986; Mosmann and Coffman, 1989). These cells were identified in the late 80s and early 90s, when it was noted that *in vitro* naïve $CD4^+$ T cells could be induced to produce IL-4 and interferon gamma (IFNγ) if they were stimulated in the presence of IL-4 and IL-12 respectively. Indeed, it was then shown that IL-4 together with IL-2 plays a strong role in Th2 differentiation and that IL-12 and IFNγ promote Th1 differentiation (Gros *et al.*, 1990; Manetti *et al.*, 1993). Th1 cell differentiation is promoted under the master regulator T-bet and functionally they are involved in the elimination of intracellular pathogens (Szabo *et al.*, 2000). Th1 cells have also been linked to organ specific autoimmunity (Dardalhon, Korn, *et al.*, 2008). Th2 cell differentiation is regulated by the transcription factor GATA-3 (Zheng and Flavell, 1997). Th2 cells build immune responses against extracellular pathogens. A persistent activation of this subset can lead to allergic diseases like asthma and atopic dermatitis (Kubo, 2017).

More recently came the discovery of the Th17, Th22, and Th9 subsets. Th17 cells were classified as an independent Th subset after the identification of the transcription factor RORγt (Harrington *et al.*, 2005; Ivanov *et al.*, 2006). TGFβ, IL-6, IL-21, and IL-23 are key cytokines for Th17 differentiation and function (Wei *et al.*, 2007; Burgler *et al.*, 2009). Important effector cytokines linked to Th17 cells include IL-17A, IL-17F, and IL-22 (Schmidt-Weber, Akdis and Akdis, 2007). This subset is involved in the elimination of extracellular pathogens including fungi and is involved in various autoimmune disorders (Zambrano-Zaragoza *et al.*, 2014). Th17 cells are recognized to be strong components in the pathogenesis of psoriasis (Lowes *et al.*, 2008). Th22 cells are characterized by secreting high amounts of IL-22 (Duhen *et al.*, 2009). They differ from other Th subsets because they do not secrete IFNγ, IL-4, IL17A and IL17-F (Eyerich *et al.*, 2009). Functionally, Th22 cells have an antimicrobial role and a role in promoting barrier integrity and wound healing. IL-22 concentrations and Th22 population are increased in atopic dermatitis and psoriasis (Dainichi *et al.*, 2018). Th9 cells were initially thought to be linked to Th2 cells, additional studies brought to the notion that they might be a distinct Th cell subset. The presence of TGFβ together with IL-4 seems to divert the differentiation process from Th2 towards Th9 (Dardalhon, Awasthi, *et al.*, 2008; Veldhoen *et al.*, 2008). These cells secrete IL-9 and play a role against helminth infection. The transcription factor IRF4 was shown to play an important role in their differentiation process (Kaplan, 2013).

Tfh were first discovered in tonsils. As their names suggest, they are found in the follicular area of secondary lymphoid organs (Crotty, 2011). Bcl6 and STAT3 are important transcription factors involved in their differentiation (Yu *et al.*, 2009) and important cytokines involved in their differentiation process include IL-21 and IL-6 (Nurieva *et al.*, 2008). They are involved in modulating the humoral response by interacting with B lymphocytes (Schaerli *et*

al., 2000). Based on the cytokines secreted by Tfh cells, they can promote different immunoglobulin responses (i.e. IgA, IgG1, IgE) (Schaerli *et al.*, 2000).

1.2.2 Regulatory $CD4^+$ Th cells

The first report of regulatory T cells was made in 1970 (Gershon and Kondo, 1970). Following this report, in 1995 Sakaguchi *et al*. reported of a subset of T cells that constitutively expressed high levels of CD25 that could suppress the immune response and inhibit from autoimmunity (Sakaguchi *et al.*, 1995). These cells are known today as regulatory T cells (Tregs). Following this discovery, much effort has been put into characterizing and better understanding this T cell subset.

$CD4^+$ Tregs are a heterogeneous population which can be further divided into different subpopulations according to the location from which they arise from and based on phenotypical features. Three subpopulations of $CD4^+$ Tregs that have been described and these are natural thymus-derived Tregs (nTregs), induced Tregs (iTregs), and inducible T regulatory type 1 (Tr1) cells (Sakaguchi *et al.*, 2008; Gregori, Goudy and Roncarolo, 2012). nTregs represent a distinct lineage directly arising from the thymus. They express FoxP3 and high levels of CD25 (Sakaguchi, 2005). Based on their activation status, they can be distinguished into naïve $CD45RA^+FoxP3^+$ nTregs and effector $CD45RA^-FoxP3^+$ nTregs (Booth *et al.*, 2010). In the periphery, $FoxP3^-CD25^-CD4^+$ naïve T cells can be stimulated to induce FoxP3 and CD25 to become iTregs. This process involves T cell receptor stimulation, IL-2 and TGFβ (Horwitz, Zheng and Gray, 2003; Zheng *et al.*, 2007). Tr1 cells represent a population of Tregs, also induced in the periphery, that lacks FoxP3 expression but can produce high amounts immunosuppressive cytokines (IL-10, and TGFβ) and low amounts of pro-inflammatory cytokines (IFNγ, IL-2, and IL-5) (Gregori, Goudy and Roncarolo, 2012).

The main function of Tregs is to maintain peripheral tolerance and hinder possible excessive immune responses. They do this by suppressing immune cells. Different mechanisms are used by Tregs to carry out this task. They can be divided into two types: direct and indirect mechanisms. Direct mechanisms include the secretion of high amounts of immunosuppressive cytokines like IL-10, TGFβ, and IL-35 (Von Boehmer, 2005). Another direct mechanism used by Tregs includes directly eliminating target cells through granzyme and perforin-mediated cytotoxicity (Cao *et al.*, 2007). Indirect mechanisms by which Tregs exert their function includes depleting available IL-2, resulting in the “starvation” of target T cells (Pandiyan *et al.*, 2007). In fact, Tregs express CD25 which can bind IL-2 and given its strong expression on the surface, it can out-compete other T cells for its uptake. Tregs can also indirectly inhibit T cell activation through the co-inhibitory molecule CTLA-4 (Read,

Malmström and Powrie, 2000) and CD39/CD37. CTLA-4 can bind the co-stimulatory molecules CD80 and CD86 expressed by APCs (Sansom, 2000) blocking CD28-mediated T cells co-stimulation (Walunas, Bakker and Bluestone, 1996). CD39/CD37 act by reducing ATP through the generation of immunosuppressive AMP (Allard *et al.*, 2017).

Given their strong immunosuppressive ability, the use of Tregs, directly through their adoptive transfer or via mechanisms to expand them *in vivo*, in the context of organ transplantation and autoimmunity have been investigated (Raffin, Vo and Bluestone, 2019). Phase I and II clinical trials have been performed where Tregs have been infused into patients that have received an organ transplant (Gregori, Passerini and Roncarolo, 2015; Gliwiński, Iwaszkiewicz-Grześ and Trzonkowski, 2017). These studies have shown the safety of adoptively transferring Tregs, however much is still to be done before they can be effectively and routinely used in the clinics. Therefore, future studies that aim to identify new strategies to expand *in vitro* and/or *in vivo* will be of great importance in this fast-evolving field.

1.3. Bone morphogenetic protein signaling

Bone morphogenetic proteins (BMP) are a sub-family of proteins that belong to the bigger transforming growth factor β (TGFβ) superfamily (Miyazono, Kamiya and Morikawa, 2010). Together with the TGFβ proteins and BMPs, also activins/inhibins and growth and differentiation factors (GDF) comprise the TGFβ superfamily (Wu and Hill, 2009). As their name indicates, much of the research done on BMPs has focused on their role in bone and osteogenesis, with studies showing that BMPs can promote bone and cartilage formation (Hogan, 1996). Later, it was shown that BMPs participate in a wide variety of processes including organ formation (i.e. heart and liver), tissue homeostasis, and vascular remodeling. In addition, BMPs have been described to also play a major role in the immune system and cancer (Chen and Ten Dijke, 2016).

BMPs are secreted as non-active precursors. The precursors become active following cleavage by convertases (Cui *et al.*, 1998). BMPs signal through heterotetrameric receptor complexes formed by type I and type II receptors. The type I receptors are seven and include: activin receptor-like kinase 1 (ACVRL1), activin A receptor, type I (ACVR1), bone morphogenetic protein receptor type IA (BMPR1a), activing receptor type-1B (ACVR1B), transforming growth factor beta receptor I (TGFβR1), bone morphogenetic protein receptor type IB (BMPR1B), and the activing A receptor (ACVR1C). The type II receptors are four and include: transforming growth factor beta receptor II (TGFβR2), bone morphogenetic protein receptor type II (BMPR2), activin receptor type-2A (ACVR2A), and activin receptor type-2B

(ACVR2B) (Miyazono, Kamiya and Morikawa, 2010). The type I and type II receptors can be shared between different ligands of the TGFβ superfamily and in addition can form different heterotetrameric complexes. These variables add to the complexity of this signaling process (Nickel and Mueller, 2019). A list of possible combinations between ligands, type I and type II receptors is provided in Table 2. Once this complex is formed, this leads to the phosphorylation of the downstream R-SMAD proteins. The R-SMAD proteins involved in BMP signaling include SMAD1, SMAD5, and SMAD8. The phosphorylated R-SMADs then bind to SMAD4 and this SMAD complex translocates to the nucleus and drives the expression of target genes.

Table 2: TGFβ ligands and receptors

Ligands	Type I receptors	Type II receptors	R-SMADs
BMPs	BMPR1a, BMPR1b, ACVR1	BMPR2, ACVR2A, ACVR2B	SMAD1/5/8
TGFβ	TGFβR1	TGFβR2	SMAD2/3
Activin A	ACVR1B	ACVR2A, ACVR2B	SMAD2/3
GDF1	ACVR1B	ACVR2A	SMAD2/3
GDF11	ACVR1B, TGFβR1	ACVR2B	SMAD2/3
Nodal	ACVR1B, ACVR1C	ACVR2B	SMAD2/3

1.3.1. Bone morphogenetic proteins in dendritic cell development and function

The role of BMPs in DC development and function has not been yet fully elucidated. Only a handful of research studies have addressed this question. DCs express BMP ligands and all the required signaling machinery (Type I and type II receptors, SMAD proteins). More and more studies are indicating that BMP signaling is involved in DC activation and function. MoDCs produce BMP4 and upon stimulation (Martínez *et al.*, 2011, 2014) it's production is further enhanced. Stimulation of moDCs with BMP4 leads to maturation with the upregulation of the surface markers CD80, CD83, CD86, PD-L1, and PD-L2 (Martínez *et al.*, 2011). Blockade of BMP signaling via the use of the BMP inhibitor dorsomorphin resulted in the downregulation of the inhibitory molecules PD-L1 and PD-L2, leaving CD80 and CD86 expression relatively unchanged. This process is mediated via the interferon regulatory factor 1 (Martínez *et al.*, 2014). These findings appear to be particularly interesting in the context of anti-tumor immunity. BMPs are also expressed in tumors (Bach, Park and Lee, 2018) and future studies may shed light on whether tumor cells may use BMPs to upregulate PD-L1 and PD-L2 expression as a means of immuneevasion.

BMPs have also been described to be involved in DC differentiation. BMP7 was shown to be able to promote LC differentiation (Yasmin *et al.*, 2013; Borek *et al.*, 2020). Prior to this, TGFβ was thought to be the essential cytokine for LC development. However, LCs precursors are present in developing skin before the active form of TGF-β1 is expressed (Schuster *et al.*, 2009). Furthermore, the addition of an inhibitor of the TGFβ type I receptor (TGFβR1) during *in vitro* LC differentiation leads to a higher number of LCs in culture, suggesting that signaling via this receptor has an interfering/anti-proliferative role in LC differentiation. This led to the discovery that TGF-β1 promotes LC differentiation via a non-constitutive pathway by signaling through BMPR1a, and that signaling via the TGFβR1 is required to keep LCs in an immature state (Yasmin *et al.*, 2013).

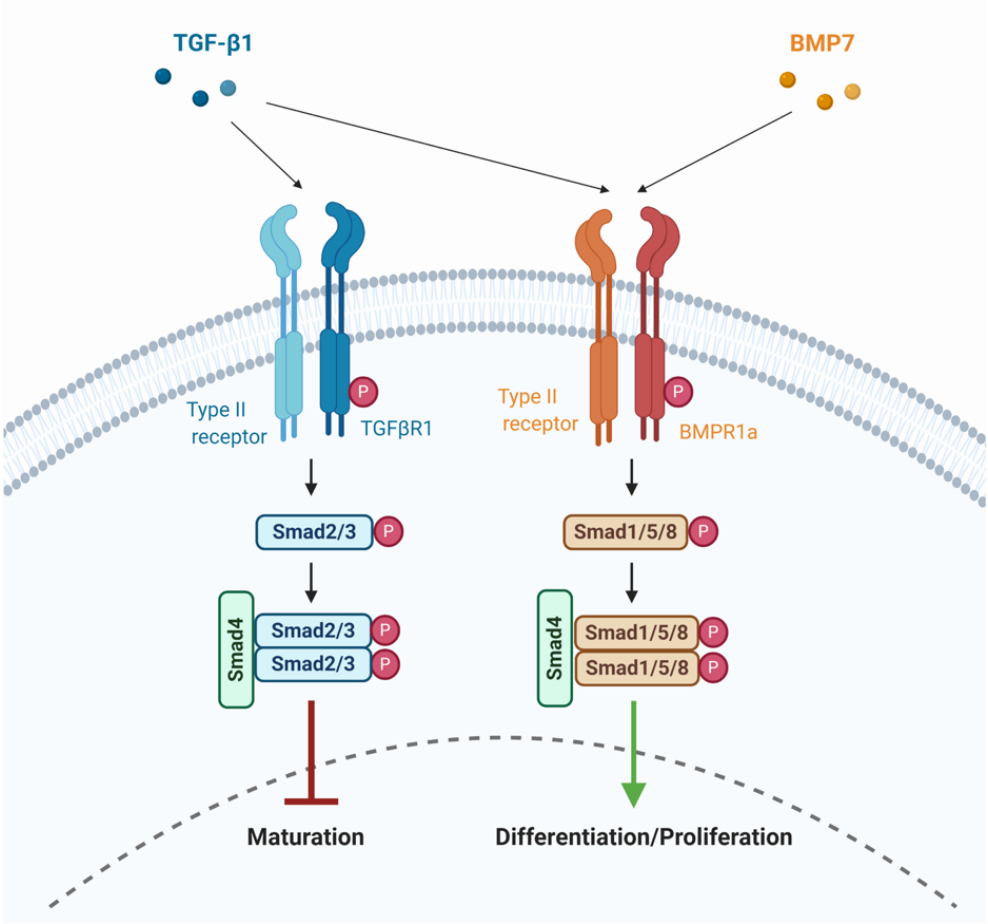

Figure 2. BMPR1a mediates LC differentiation. Other than its constitutive pathway, which involves signaling through TGβR1, TGF-β1 can also signal through the non-constitutive pathway which involves binding to BMPR1a. In LC biology, signaling via BMPR1a promotes LC differentiation and proliferation, whereas signaling via the constitutive pathway inhibits the maturation of LCs and is responsible for maintaining LCs in an immature state. This figure was designed with BioRender.com.

1.3.2. Bone morphogenetic proteins in T cell development, homeostasis, and function

Subsequent studies showed that BMPs play an important role in thymus organogenesis, T cell development, homeostasis and function. BMP signaling in the thymus participates in T cell development. BMP2 and BMP4 are produced within the thymus, mostly by the thymic stroma (Hager-Theodorides *et al.*, 2002). Inhibition of BMP signaling in thymic cells leads to the development of a smaller thymus and a slower occupation of the thymus by lymphoid progenitors (Bleul and Boehm, 2005). During T cell development, BMP4 is involved in the DN1 stage and the stage in which $CD4^{-}CD8^{-}$ double negative cells transition into

$CD4^+CD8^+$ double positive cells, blocking their differentiation at these stages (Tsai, Lee and Wu, 2003). BMP signaling is also involved in the homeostasis and function of mature T cells. The first studies that investigated its role in these processes used dorsomorphin (DM). DM is a small molecule inhibitor of the BMP signaling pathway that acts on ACVR1, BMPR1a, and BMPR1b. These studies showed that BMP signaling is important for naïve $CD4^+$ T cell activation. Following anti-CD3/CD28 activation, naïve $CD4^+$ T cells upregulate the BMPR1a receptor and BMP2, 4, and 6 were induced (Martínez *et al.*, 2015). In addition, BMP signaling regulates IL-2 production and the expression of the IL-2 receptor (CD25) by T cells, and their treatment with DM leads to an impaired production of IL-2 and decreased expression of CD25 (Yoshioka *et al.*, 2012). Also by using DM *in vitro,* it was shown that BMP signaling affects naïve $CD4^+$ T cell polarization, more specifically regarding Th17 and Treg differentiation. Naïve $CD4^+$ T cells cultured in the presence of DM, do not differentiate into Th17 cells and Tregs as efficiently as when they are cultured under normal Treg polarizing conditions (Yoshioka *et al.*, 2012). Supporting the role of BMPs in Treg differentiation, it was also shown that BMP2 and BMP4 synergistically act with TGF-β1 to more efficiently promote Tregs (Lu *et al.*, 2010). More recently, the effect of BMP signaling in priming $CD4^+$ T cells was also investigated *in vivo*. To do so, conditional knockout (KO) mice were bred to specifically knockout the BMPR1a receptor in $CD4^+$ T cells. Under steady-state conditions, the mice were phenotypically identical, but expressed lower percentages of Tregs. In addition, naïve $CD4^+$ T cells isolated from the KO mice did not differentiate efficiently into Tregs when cultured under Treg polarizing conditions (Kuczma, Kurczewska and Kraj, 2014). The same group, also showed that naïve $CD4^+$ T cells from BMPR1a-KO mice differentiate more easily into Th17 cells, and that the addition of BMPs to Th17 polarizing cultures blocks Th17 differentiation, in contrast with what was previously shown in the *in vitro* DM experiments (Browning *et al.*, 2018). However, this may be because DM inhibits three different TGFβ type I receptors, whereas in the KO mouse only BMPR1a was affected. The *in vivo* experiments, also showed that this signaling pathway in T cells plays a strong role in the progression of autoimmune diseases and cancer. Indeed, BMPR1a deficient $CD4^+$ T cells blocked more efficiently the growth of melanoma cells when these were adoptively transferred to the KO mice compared to the wild-type (WT) mice. This effect may be due to the lower amounts of Treg cells present in these mice, rendering the conventional T cells more suitable in immunesurveillance (Kuczma, Kurczewska and Kraj, 2014). BMPR1a-KO mice also experience a more exacerbated colitis in the DSS-induced inflammatory bowel disease model, with a strong Th17 signature (Browning *et al.*, 2018). The studies performed so far clearly indicate an important role for BMP signaling in T cells, and connected diseases like gastrointestinal inflammatory diseases or cancer. Future studies should aim to elucidate

whether this signaling pathway is also involved in other disease models and in other cell types and whether targeting it specifically may represent valid a therapeutic option.

1.4. Psoriasis

Psoriasis is a chronic skin inflammatory disorder. Its prevalence is quite common and it affects around 1-3% of the population worldwide. Macroscopically, this disease is characterized by red plaques. The most affected parts of the body include the scalp, elbows, and knees, however, depending on the severity of the disease it can affect any part of the body. The interested parts can also appear dry, itchy, and scaly (Nestle, Kaplan and Barker, 2009). Moreover, depending on the severity, it can manifest itself from very small spots to large areas. The involved areas can go through phases, first manifesting themselves, then healing, and then coming back again throughout the lifespan of the patient. Histologically, psoriasis is characterized by keratinocyte hyper proliferation and premature maturation, leading to a thickening of the epidermal compartment of the skin. Different forms of psoriasis are known, but the form that is most commonly referred to is *Psoriasis vulgaris*. Around 90% of the psoriasis cases described refer to this form (Griffiths and Barker, 2007). Psoriasis cannot be transmitted between people, but it can manifest itself more frequently between members of the family, suggesting a genetic predisposition for this disease (Elder, Nair and Voorhees, 1994). There is currently no cure for psoriasis, the treatment of the disease is based on a symptomatic treatment to slow down or ameliorate the symptoms. Early therapeutic strategies consisted in the use of topical and systemic immunosuppressive drugs like corticosteroid-based creams, methotrexate, and cyclosporine. With the introduction of monoclonal antibody-based therapies, a new and strong tool has been introduced in the treatment of psoriasis. One of the first monoclonal antibodies to be used was one that acts by blocking TNFα. This strategy proved to be particularly effective and different TNFα blocking antibodies have been approved for use in the clinics. More recently approved monoclonal antibodies used in the treatment of psoriasis include antibodies that act by blocking IL-17A and IL-23 (Kim and Krueger, 2017).

1.4.1. The role of the immune system in psoriasis

In psoriasis, the immune system plays a strong role in the pathogenesis of the disease. In fact, early studies identified strong immune cell infiltration in skin lesions of psoriasis patients. Following this observation, other studies promoted the notion that the interplay between the components of a dysregulated immune system has a functional link in the pathogenesis of psoriasis. These observations include for example 1) the appearance of clonal T cells over time in the lesions of psoriasis patients, 2) the fact that following bone marrow

transplantation some patients are cured from psoriasis, and 3) that newly developed biological therapies (i.e. monoclonal antibodies), which target pro-inflammatory cytokines, have proven to be very effective in ameliorating psoriatic lesions. Two components of the immune system that are strongly implicated in the pathogenesis of psoriasis are DCs and T cells.

During psoriasis, there is a strong accumulation of dDCs, with increases that can reach up to 30 times the amounts that are present in normal skin. This suggests a role of inflammatory dDCs in the pathogenesis of psoriasis. These DCs can interact with T cells promoting their proliferation and activation. As mentioned earlier (section 1.1.1.), these inflammatory DCs include Tip-DCs and slan-DCs which present the ability of producing iNOS and TNFα. Another characteristic of inflammatory dDCs that contributes to psoriasis, is their ability to secrete IL-23. IL-23 is a DC-derived cytokine that can stimulate $CD4^+$ T cell to secrete IL-17A and IL-22, which has been shown to have a strong role in psoriasis. Another DC subset, pDCs, are involved in psoriasis. In healthy skin, pDCs are practically absent or present in very low amounts. In psoriasis, the numbers of pDCs in the skin increases, and studies have shown that there is a strong type I IFN signature in psoriasis and that pDCs are involved in the initiation phase of the disease (Glitzner *et al.*, 2014). Instead, the role of LCs in psoriasis remains not so clear cut, with studies that report findings that may also be contradictory with one-another. LCs isolated from psoriatic skin lesions present the ability to secrete IL-23 following toll-like receptor (TLR) stimulation (Martini *et al.*, 2017), which plays part in the IL-17A/IL-22/IL-23 axis that is involved in psoriasis. Contrariwise, LCs from psoriatic lesions show increased expression of certain tolerogenic factors like PD-L1, PD-L2, and IDO-1 (Martini *et al.*, 2017). Data obtained from murine models of psoriasis-like inflammation also differ. Data from mice that were treated with imiquimod to induce a psoriasis-like skin inflammation show that LCs are activated and produce IL-23 contributing to the onset of psoriasis. Instead, data obtained from $Junf^{fl/fl}JunB^{fl/fl}K5cre^{ER}$ (DKO*) mice, show that LCs express higher levels of IL-10 mRNA and PD-L1, and that their depletion leads to an exacerbation of the disease (Glitzner *et al.*, 2014). This indicates that the role of LCs is not so black and white and much is still needs to be elucidated.

T cells are very important in the pathogenesis of psoriasis. In the beginning, it was described that psoriatic lesions present an environment with a strong Th1 and $CD8^+$ cytotoxic T cell signature with high levels of TNFα, IFNγ, IL-12. Later, with the discovery of Th17 subset, it was also shown that this Th subset is actively involved in this disease. Following this discovery, it was shown in mice that the IL-17A/IL-22/IL-23 axis is required for the onset of symptoms in imiquimod-induced psoriasis-like inflammation, remarking the influence of Th17 cells in psoriasis and challenging the view that psoriasis is a Th1 mediated disease. In addition,

the selective inhibition of the IL-17 signaling has proven to have good results in treating psoriatic lesions. Another T cell population that plays a role in psoriasis are Tregs. Initial studies described Tregs in lesions of psoriasis patients to be “defective”, in the sense that they are not able to suppress effector T cells, allowing for their un-controlled proliferation and enhanced inflammation. It was also described that Tregs isolated from psoriatic lesions can be easily converted into Th17 or $IL17^{+}FoxP3^{+}$ T cells (Bovenschen *et al.*, 2011). This suggests a strong plasticity of this subset, and that under severe inflammatory conditions, they might be instructed to differentiate into pro-inflammatory T cells thus directly contributing to the disease. More recent studies, however, also described an active suppressive role of Tregs during psoriasis-like inflammation. These studies were conducted in mice and imiquimod was used to induce skin inflammation. Depletion of Tregs led to and exacerbated skin inflammation mediated by an uncontrolled $CD4^{+}$ T helper response (Hartwig *et al.*, 2018) and an enhanced $CD8^{+}$ T cell response (Stockenhuber *et al.*, 2018). These studies proved that Tregs play are functionally active in psoriasis and play an important role by putting a brake on the inflammatory response. The mechanisms that regulate the function of this T cell subset in psoriasis is still not understood and future studies that aim at understanding these mechanisms could open new therapeutic options by figuring out ways to expand them or enhance their suppressive power in the lesions.

2. Aim of the study

DCs are professional APCs responsible for orchestrating and regulating the immune response with the purpose of clearing undesired pathogens and maintaining tolerance, thus playing a central role in the maintenance of homeostasis. Different DC subsets have been described, and the DCs that populate the epidermis are referred to as LCs. They form a packed network and due to their proximity to the external environment they are continuously exposed to various danger signals. Recent studies described the importance of LCs in maintaining immune tolerance during steady-state and this is mediated by their ability to promote the proliferation of skin resident FoxP3$^+$Tregs. In addition, mouse LC depletion studies described a possible regulatory role for LCs during skin inflammation.

The epidermis, by releasing various cytokines and signals during steady-state and inflammation, can alter LC function. Gene profiling of murine skin during inflammation revealed that the highest induced genes are *TSLP*, *TGFB1*, and *BMP7*. Consistent with the above described profiling, previous work from our laboratory described that BMP7 is overexpressed during psoriasis. Normally, under healthy conditions, BMP7 expression is confined to the suprabasal KC layer in the epidermis. However, in conditions such as psoriasis, it's expression is no longer limited to this area but expressed throughout the whole epidermis. Both pro-inflammatory and inflammatory roles have been attributed to BMP7, however, it's role in skin inflammation is not completely understood.

Additionally, BMP7 is involved in LC differentiation. BMP7 can induce *in vitro* LC differentiation from CD34$^+$ HPCs. BMP7 promotes the generation of LCs that are phenotypically diverse from TGF-β1-driven LCs and that resemble LCs that can be found in psoriatic lesions (CD207$^+$CD206$^+$CD1c$^+$TLR2$^+$). Preliminary studies described these cells to secrete higher amounts of cytokines following stimulation with the TLR2 agonist peptidoglycan in comparison to TGF-β1-LCs. However, their role during skin inflammation is still to be elucidated.

Given the aberrant expression of BMP7 in the epidermis of psoriatic lesions and its role in promoting psoriatic-like LCs, the aim of our investigation was to study the effects of BMP7 and BMP receptor signalling on DC/LC function and their role in regulating psoriasis-like skin inflammation.

3. Materials and methods

3.1. Primary cell isolation

3.1.1. CD34$^+$ hematopoietic progenitor cell isolation

Cord blood was collected during healthy, full-term deliveries. Ethical approval (EK 26-520 ex 13/14) was obtained from the Medical University of Graz. From the umbilical cord blood, blood mononuclear cells (MNCs) were isolated by density gradient separation with Lymphoprep (Axis Shield, Oslo, Norway). Cord blood was diluted (1:2) with 1X PBS. In a 50 ml tube, 20 ml of blood was gently placed on top of 20 ml of Lymphoprep. Following a 30 min centrifugation phase (no brake, 1400 rpm), the interphase was collected and cells were washed with 1X PBS (low brake, 1600 rpm). Red blood cells were then lysed by re-suspending the cell pellet in 1 ml ACK lysis buffer and cells were left for 10 min on ice. Following the 10 min incubation, the tubes were filled with 1X PBS and centrifuged for 20 min (low brake, 700 rpm). Subsequently, MNCs were counted and suspended at the desired concentration. From the MNCs, CD34$^+$ cells were isolated by magnetic sorting using EasySep human CD34 positive selection kit (Stem Cell Technologies, Vancouver, Canada) according to manufacturer's protocol. Purity was checked by flow cytometry. CD34$^+$ cells were then cultured for 3 days in serum-free X-Vivo 15 media supplemented with Glutamax (Lonza, Basel, Switzerland), penicillin/streptomycin, 50 ng/ml SCF, 50 ng/ml FLT3L, and 50 ng/ml TPO.

3.1.2. Naïve CD4$^+$ T cell and CD14$^+$ monocyte isolation

Buffy coats were purchased from the Transfusion Medicine Department of the Medical University of Graz, Austria. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation as described above. From PBMCs, naïve CD4$^+$ T cells were isolated by negative selection using the MagniSort human CD4 naïve enrichment kit (ThermoFisher, Waltham. MA, USA) according to manufacturer's protocol, and CD14$^+$ monocytes were isolated by positive selection (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's protocol.

3.2. *In vitro* differentiation cultures

Cytokines and reagents used for the *in vitro* differentiation protocols are listed in Table 3.

3.2.1. LC differentiation

Umbilical cord blood isolated $CD34^+$ cells were suspended at a concentration of $5x10^4$ cells/ml and cultured for 7 days in 24 well tissue treated plates (1 ml per well) in serum free CellGroDC medium (Cellgenix, Freiburg, Germany) supplemented with Glutamax, penicillin/streptomycin, TNFα (2.5 ng/ml), GM-CSF (100 ng/ml), FLT3L (50 ng/ml), SCF (20 ng/ml), and TGF-β1 (1 ng/ml), to obtain TGF-β1-LCs, or BMP7 (200 ng/ml), to obtain BMP7-LCs. For BMP7-LCs, cells were split twice, on the fourth day and the sixth day of culture. After 7 days, total cells were collected and enriched by positive magnetic selection by using anti-CD207 mAbs (Miltenyi Biotec).

3.2.2. MoDC differentiation

$CD14^+$ monocytes were suspended at a concentration of $1x10^6$ cells/ml and cultured 6 well tissue treated plates (2 ml per well) in RPMI-1640 supplemented with 10% FBS (Sigma-Aldrich, St. Louis, Mo, USA), Glutamax, penicillin/streptomycin, GM-CSF (100 ng/ml), and IL-4 (35 ng/ml). On day 4, 1 ml of old culture media is replaced with 1 ml of fresh media.

3.2.3. Treg differentiation

Before starting, 48-well suspension cell plates were coated with anti-CD3 mAb. To coat the wells, 500 μl of a PBS solution containing 3 μg/ml of anti-CD3 mAb were added to each well. Plates were incubated at 37°C/ 5%CO_2 for 4h. Before use, the antibody solution was removed and wells were washed with 500 μl of 1X PBS. Naïve $CD4^+$ T cells were suspended at a concentration of $2x10^6$ cells/ml and cultured for 4 days in 48 well suspension cell plates with immobilized anti-CD3 mAb (3 μg/ml) in serum-free X-Vivo 15 media supplemented with Glutamax, penicillin/streptomycin, IL-2 (50 μg/ml), and TGF-β1 (3 ng/ml).

Table 3: Cytokines and reagents

Cytokines and reagents	Company
Human bone morphogenetic protein 7 (BMP7)	Immunotools (Friesoythe, Germany)
Human tumor necrosis factor-a (TNFα)	Peprotech (London, UK)
Human thrombopoietin (TPO)	Peprotech
Human stem cell factor (SCF)	Peprotech
Human FMS-related receptor tyrosine kinase 3 ligand (FLT3L)	Peprotech
Human granulocyte-macrophage colony stimulating factor (GM-CSF)	Peprotech
Human interleukin-4 (IL-4)	Peprotech
Human interleukin-2 (IL-2)	Peprotech
Human transforming growth factor-β1 (TGF-β1)	R&D systems (Minneapolis, MN, USA)
Recombinant human BMPR-IA/ALK3 Fc chimera	R&D systems
SB431542	TOCRIS (Bristol, UK)
Dorsomorphin (DM)	TOCRIS
Ultra-LEAF anti-CD3ε (OKT3 clone)	Biolegend (San Diego, CA, USA)
Ultra-LEAF anti-CD28 (CD28.2 clone)	Biolegend
Treg suppression inspector beads	Miltenyi Biotec (Bergisch Gladbach, Germany)
Cell activation cocktail (without Brefeldin A)	Biolegend
Brefeldin A	Biolegend
Violet proliferation dye 450	BD Biosciences
CFSE	BD Biosciences

3.3. Mixed leukocyte reaction (MLR)

Magnetically sorted $CD4^+CD45RA^+$ naïve T cells ($1x10^5$) were cultured in 96-well U-bottom plates with either LCs or moDCs ($2.7x10^4$). After 5 days of culture, T cells were analyzed by flow cytometry for surface markers expression or intracellular cytokines. When proliferation was analyzed, naïve $CD4^+$ T cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) as follows: cells were washed and suspended in 100 μl RPMI-1640 without serum, then add 10 μM of CFSE to the cell suspension and quickly vortex. Incubate at room temperature for 5 min in the dark. Wash cells two times with RPMI-1640 media containing serum and suspend at the concentration of $1x10^6$ cells/ml. CFSE-labeled naïve T cells ($1x10^5$) were then cultured with increasing concentrations of LCs ($3x10^2$ – $2.7x10^4$) for 5 days.

3.4. Suppression assay

After the co-culture of naïve T cells with LCs, T cells were isolated by magnetically depleting LCs with anti-CD207 mAbs and rested in RPMI-1640 media supplemented with 10% FCS, Glutamax and penicillin/streptomycin. After 2 days, T cells were collected, washed and labeled with the violet proliferation dye 450 (BD biosciences) as follows: cells were washed and suspended in 1 ml pre-warmed PBS, 3 μM of the dye were added and cells were quickly vortexed. Subsequently, cells were incubated at 37°C for 10 min in the dark, washed 2 times with RPMI-1640 with serum and suspended at a concentration of $1x10^6$ cells/ml. Increasing concentrations of violet labeled T cells were added to a fixed amount of CFSE-labeled autologous $CD4^+$ responder T cells and stimulated with Treg suppression inspector beads (1:1 ratio). After 4 days, responder T cell proliferation was measured by flow cytometry. Suppression was calculated by using the following formula: (% of proliferation of Tresp alone - % of proliferation of Tresp treated with polarized T cells)/ % of proliferation of Tresp alone) *100.

3.5. Flow cytometry

Cells in 50 μl of staining buffer were stained with fluorescent-labeled antibodies. Flow cytometry antibodies used are described in Table 4. To block Fc receptors, cells were incubated for 10 mins at 4°C with human serum. To detect intracellular FoxP3 expression, FoxP3 staining buffer set (ThermoFisher Waltham. MA, USA) was used according to manufacturer's protocol. To detect intracellular cytokines, cells were first fixed in 100 μl of

Fixation Buffer A and afterwards permeabilized in 100 μl of Permeabilization Buffer B (Nordic MUbio, Susteren, Netherlands). The LSRFortessa (BD Biosciences) was used to measure and record samples. The recorded flow cytometry data was analyzed by using the DIVA (BD Biosciences) and FlowJo software (Tree Star, Ashland, OR, USA).

Table 4: Flow cytometry antibodies

Antibody	Reactivity	Clone	Fluorophore	Company
Anti-CD1a	Human	HI149	BV421 and APC	BD Biosciences
Anti-CD4	Human	RPA-T4	PE	BD Biosciences
Anti-CD25	Human	2A3	BV421	BD Biosciences
Anti-CD14	Human	M5E2	APC	BD Biosciences
Anti-IFNγ	Human	B27	FITC	BD Biosciences
Anti-CD80	Human	L307.4	AF700	BD Biosciences
Anti-CD86	Human	FUN-1	PE-Cy7	BD Biosciences
Anti-HLA-DR	Human	G46-6	FITC	BD Biosciences
Anti-CD324	Human	67A4	AF647	BD Biosciences
Anti-PD-L1	Human	29E-2A3	PE-Cy7	Biolegend
Anti-PD-L2	Human	24F.10C12	AF647	Biolegend
Anti-CD11b	Human	ICRF44	PE and PE-Cy7	Biolegend
Anti-IL-17A	Human	BL168	PE-Cy7	Biolegend
Anti-IL-4	Human	MP4-25D2	PE-Cy7	Biolegend
Anti-IL-10	Human	JES3-9D7	BV421	Biolegend
Anti-IL-2	Human	MQ1-17H12	FITC	Biolegend
Anti-CD207	Human	DCGM4	PE	Beckman Coulter
Anti-CD3	Human	Leu 4	FITC	BD Biosciences
Anti-IL-22	Human	IL22JOP	APC	eBioscience
Anti-FoxP3	Human	236A/E7	APC	eBioscience

3.6. Cytokine Measurements

TGFβ superfamily proteins were measured by using the Quantibody® Human Bone Metabolism Array 1000 (RayBiotech, Norcross, GA, USA), and the human Quantikine BMP7 ELISA kit (R&D Systems, Minneapolis, MN, USA). Other cytokines were measured using Luminex system (Luminex Corp, Austin, Texas, USA) according to manufacturer's protocol.

3.7. RNA isolation and real-time PCR.

$5x10^5$ cells were pelleted and RNA isolation was isolated by using the RNeasy® Micro Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA concentrations and purity were checked on the Nanodrop ND-1000. 1-2 μg of isolated RNA was then reverse-transcribed into cDNA by using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems by ThermoFisher) according to manufacturer's protocol. mRNA levels of target genes were analyzed via real-time PCR (qPCR) using the QuantiTect® SYBR® Green PCR kit (Qiagen) in a C1000™ Thermal Cycler (Biorad, Minneapolis, MN, USA). GAPDH was used as housekeeping gene and the relative expression of target genes was calculated by normalizing their expression to GAPDH. List of primers and their respective sequences used for qPCR are listed in the Table 5.

Table 5: Real-time PCR primer sequences

Gene	Orientation	Sequence
ACVR1A	fwd	TTG GAG ACA GCA CTT TAG CAG
	rev	GCG AGC CAC TGT TCT TTG T
BMPR1A	fwd	GAG TTG CTG CAT TGC TGA C
	rev	GAG CCA TGT AGC GTT TGG T
ACVR1B	fwd	TGC AAC AGG ATC GAC TTG A
	rev	ATG ATG CCT ACC AGC TCC A
TGFBR1	fwd	AGG CCA AAT ATC CCA AAC AG
	rev	TAG CTG CTC CAT TGG CAT AC
BMPR1B	fwd	AAG CCA GCT GGT TCA GAG A
	rev	AGG ACC CTG TCC CTT TGA T
ACVR1C	fwd	CGA ATG CTG CTT CAC AGA T
	rev	ATA ATG ATG GCC AGC TCC A
GAPDH	fwd	CCT TCC GTG GAG AAA CCT C
	rev	TGA GAG GCG GGA AAG TTG

fwd, Forward*; rev,* Reverse

3.8. Patient samples

Paraffin-embedded materials from biopsy samples were obtained from six patients participating in a clinical study investigating the effect of topical dithranol in psoriasis. The patients are 5 males and 1 female with a median age of 54.5 years (range 21.2-76.9 years. Dithranol study Clinical Trials.gov no. NCT02752672; approval number A23/15, Ethical Committee of the State of Carinthia, Austria. Informed consent was provided to patients in accordance with the Declaration of Helsinki.

3.8.1 Immunofluorescence on human samples

5 µm paraffin sections were deparaffinized in xylene, and rehydrated with decreasing concentrations of ethanol according to standard method. Sections were subjected to HIER antigen retrieval in Target Retrieval Solution pH 6.0 (Agilent/Dako, USA) for 10 min in a domestic microwave oven. Slides were cooled for 45 min at room temperature before rinsing in Tris-Buffered Saline Tween-20 (TBST, pH 7.4). All incubation steps were performed in a dark moist chamber at room temperature. Negative controls were incubated with the appropriate IgG fractions as isotype controls. Sections were blocked with 5% donkey serum-5% BSA in TBST for 1 hour prior to primary antibodies. Sections were incubated overnight at 4°C. After 10 min of TBST wash, secondary antibodies were applied for 30 min. After rinsing in TBST, DAPI was added to the slides for 15 min as a nuclei counter stain, then rinsed again with TBST before mounting with Dako Fluorescence Mounting Medium (Agilent, Inc., Santa Clara, CA). Primary and secondary antibodies can be found described in Table 6.

3.9. Mouse experiments

To generate DC-specific BMPR1a knockout mice (BMPR1aΔCD11c), $BMPR1A^{losP}$ mice were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA) and crossed with CD11c-cre mice on a C57BL/6 background at the Medical University of Vienna in the animal facility. BMPR1a deletion was checked through PCR. The mice were housed under specific-pathogen-free conditions in the animal facility of the Medical University of Vienna in agreement with institutional policies and federal guidelines. Experimental procedures were approved by the Animal Experimental Ethics Committee of the Medical University of Vienna and the Austrian Federal Ministry of Science and Research (animal license numbers: GZ 66.009/124-BrGT/2003, GZ 66.009/109-BrGT/2003, GZ BMWF-66.009/0073-II/10b/2010, GZ BMWF-66.009/0074-II/10b/2010, GZ BMWFW-66.009/0200-WF/II/3b/2014, and GZBMWFW-66.009/0199-WF/II/3b/2014).

3.9.1 Psoriasis-like skin inflammation model

Psoriasis like skin inflammation was induced by using the TLR7 agonist imiquimod. Imiquimod (circa 2 mm^3) was applied on the dorsal side of the mice's ears daily for 7 days. Ear thickness was measured via caliper every day. The relative change in ear thickness was measured by normalizing the ear thicknesses of every mouse with the respective ear thickness on day 0 of the experiment. To collect samples, mice were euthanized by cervical dislocation at the indicated time points.

3.9.2. Epidermal sheets

Mouse ears were separated, and floated on ammoniumthiocynate for 20-25 min. at 37°C. Then, the epidermis was peeled off, fixed in 4% PFA for 30 min at room temperature, washed in PBS, and then stained with fluorescent antibodies (anti-MHCII-PE 1:300, anti-CD207-FITC 1:300, HOECHST 1:1000) for 30 min. in the dark at room temperature. Images were taken at 10X, and LCs were counted manually. Per epidermal sheet, five pictures were taken and one high power field (HPF) per picture was counted. Each data point represents the mean of five HPF.

3.9.3. Immunofluorescence on mouse samples

4 μm cryosections of mouse skin samples were prepared and used for immunofluorescence studies. Prior to tissue fixation, sections were taken out from -20°C and warmed to room temperature for 20 minutes. Tissue sections were fixed in 4% paraformaldehyde (PFA) for 10 minutes and rinsed in double distilled water prior to immunofluorescent staining. Sections were washed with TBST, blocked with 5% donkey serum for 30 minutes before primary antibody, and were then incubated at 4°C overnight. All incubation steps were performed in a dark moist chamber at room temperature. After 10 minutes of TBST wash, the secondary antibody was applied for 30 minutes. Following another TBST wash, DAPI (5 μg/ml) was added to the slides for 15 minutes as a nuclei counter stain. Sections were rinsed again with TBST before mounting with Vectashield mounting medium (Vector Lab, Inc.). Primary and secondary antibodies can be found described in Table 6.

Table 6: Immunofluorescence antibodies

Antibody	Reactivity	Fluorophore	Clone	Company
Primary rat anti-FoxP3	Human	-	PCH101	Invitrogen
Primary rabbit anti-BMP7	Human and mouse	-	Polyclonal	LSBio
Primary rabbit anti-pSMAD1/5/8	Human	-	Polyclonal	Cell Signaling
Primary rat anti-FoxP3	Mouse	-	FJK-16s	ThermoFisher
Secondary donkey anti-rat Cy-3	Rat	Cy-3	H+L	Jackson Immuno Lab
Secondary donkey anti-rabbit	Rabbit	AF647	H+L	Jackson Immuno Lab

3.10. Image acquisition

Images of sections were acquired and analyzed by using a Leica DM4000 B microscope (Leica Cambridge Ltd) equipped with Leica DFC320 Video camera (Leica Cambridge Ltd).

3.11. Statistical analysis

To determine the statistical significance of the experiments GraphPad6 (GraphPad Software Inc, San Diego, CA, USA) was used. To compare two groups, the two-tailed Student's t-test was used. Analysis of variance (ANOVA) followed by post hoc tests were used to compare the statistical significance between more than two groups. Statistical significance was considered when P values <0.05 were calculated.

4. Results

4.1. BMP7 and FoxP3$^+$ Tregs are increased in psoriatic lesions and positively correlate

Tregs were recently described to play an active role in psoriasis-like inflammation in mice by accumulating in psoriatic lesions in order to regulate the severity of the inflammatory process (Hartwig *et al.*, 2018; Stockenhuber *et al.*, 2018). We began our study by investigating whether this can also be seen in human psoriatic lesions. Comparison between non-lesional and lesional skin biopsies taken from six patients with psoriasis revealed that the number of Tregs is significantly increased in psoriatic lesions (6.1 ± 2.5 FoxP3$^+$ cells/mm^2 vs 93.9 ± 43.27 FoxP3$^+$ cells/mm^2) (Figure 3).

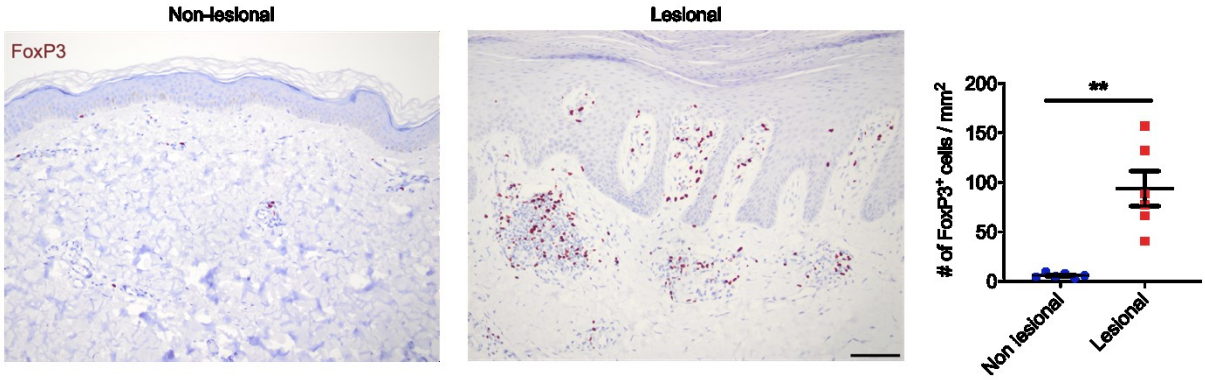

Figure 3. FoxP3$^+$ cells accumulate in psoriatic skin lesions. Biopsies from non-lesional and lesional skin of psoriasis patients were stained for FoxP3 to asses Treg numbers. Representative high power field of skin biopsies showing the presence of FoxP3$^+$ stained cells (left). The scale bar represents 100 μm. The graph shows the number of FoxP3$^+$ cells per mm^2 (right). Data are shown as mean ± SEM (n = 6) and were analyzed by using a two-tailed paired Student's t-test. **P<0.01. Figure published in (Sconocchia *et al.*, 2020).

BMP7 and downstream BMP signaling are aberrantly enhanced during psoriasis. In healthy human skin, BMP7 expression in confined to the suprabasal epidermal layer, whereas during psoriasis, BMP7 is no longer restrained to this area but is expressed throughout the entire epidermis (Borek *et al.*, 2020). We therefore asked whether there could be a connection between BMP7 levels and Tregs in the psoriatic lesions. As expected, when comparing the

lesional with the non-lesional psoriatic skin, BMP7 expression is no longer confined to the suprabasal epidermal layer but spread throughout its entirety (Figure 4A).

Psoriatic skin lesions expressed significantly higher BMP7 staining intensity compared to non-lesional skin form the same patient (Figure 4B). Interestingly, even though the number of Tregs and BMP7 staining intensities substantially varied between the patients, these two positively correlated in the psoriatic skin lesions. (Figure 3C).

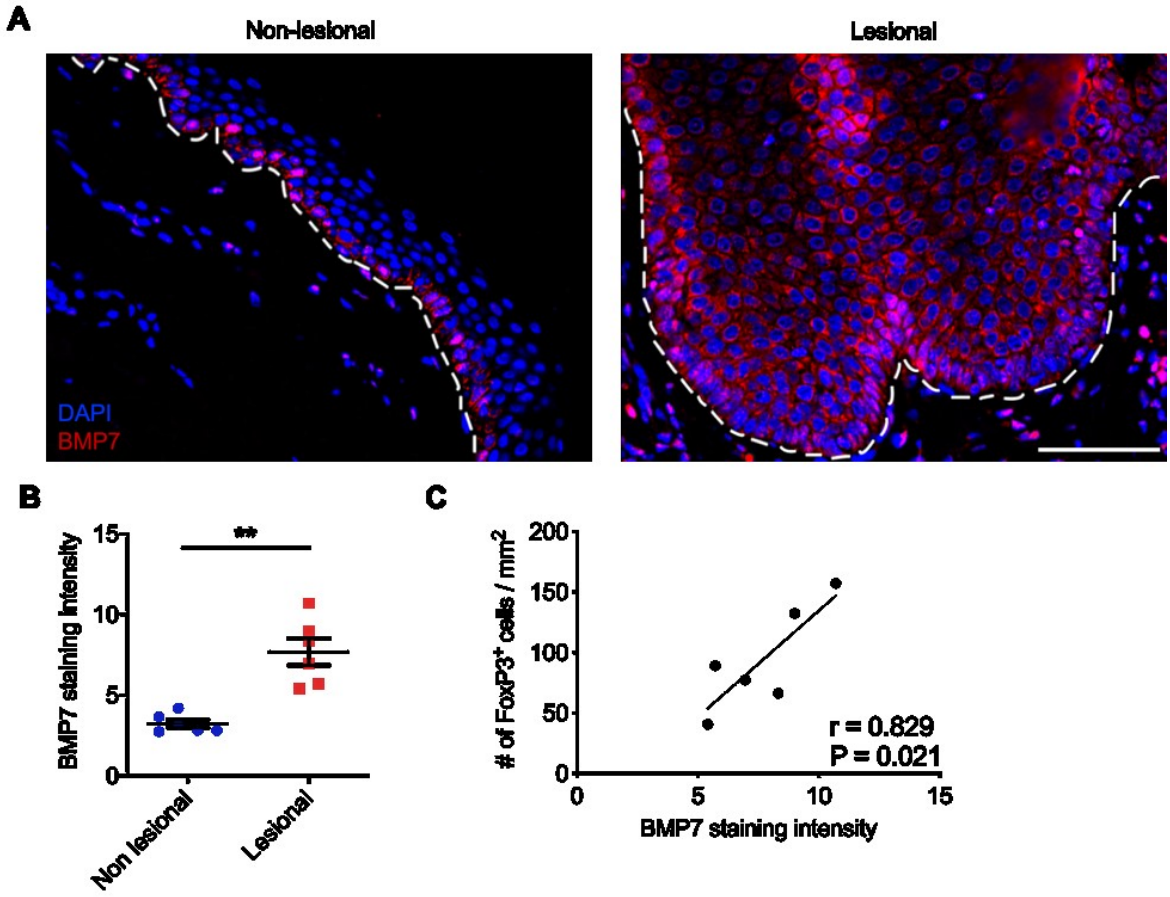

Figure 4. BMP7 intensity positively correlates with FoxP3$^+$ cell numbers in psoriatic skin lesions. Biopsies from non-lesional and lesional skin of psoriasis patients were assessed for BMP7 expression. (A) Representative immunofluorescence staining of BMP7 (red) in skin biopsies. DAPI was used to stain nuclei. The white-dotted line indicates the border between the dermis and epidermis. Scale bar represents 50 μm. (B) Graph showing the BMP7 staining intensity calculated using ImageJ software. Data are shown as mean ± SEM (n = 6) and were analyzed using a two-tailed paired Student's t-test. **P<0.01. (C) Graph showing the correlation between the number of FoxP3$^+$ cells per mm^2 in lesional skin and BMP7 staining

intensity. Each dot represents a patient (n = 6). The data were analyzed using Pearson's correlation. Figure published in (Sconocchia *et al.*, 2020).

4.2. TGF-β1-LCs and BMP7-LCs are phenotypically and functionally different

TGF-β1 is the most known and studied factor involved in LC differentiation. BMP7 was recently identified as an instructive factor for LC differentiation and was shown to be able to promote LC differentiation from CD34⁺ hematopoietic progenitor cells (HPCs) in substitution of TGF-β1 (Yasmin *et al.*, 2013). BMP7-differentiated LCs (BMP7-LCs) express the typical LC markers like CD1a and CD207, however a more thorough analysis revealed that they express a unique marker profile in comparison to TGF-β1-differentiated LCs (TGF-β1-LCs), by expressing markers such a CD206 and CD1c (Borek *et al.*, 2020) (Figure 5A).

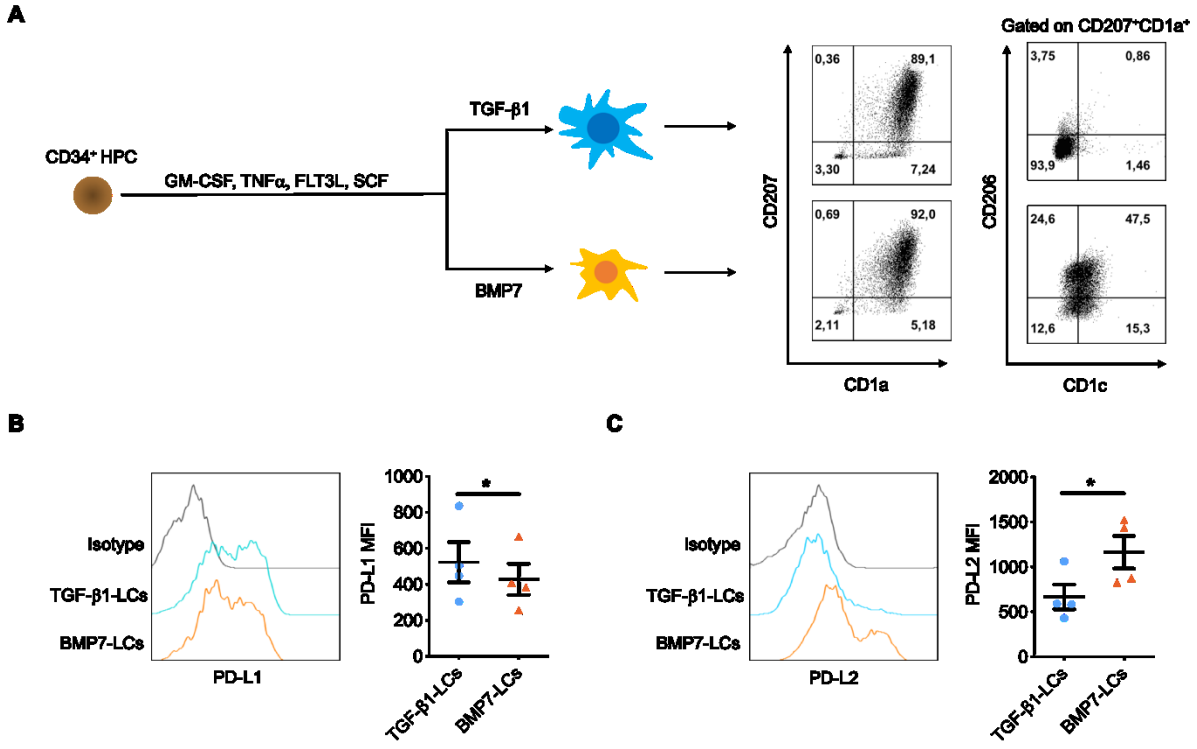

Figure 5. BMP7-LCs have a unique marker profile and are stronger at promoting T cells proliferation in comparison to TGF-β1-LCs. TGF-β1-LCs and BMP7-LCs were differentiated *in vitro* by culturing CD34⁺ HPC in the presence of GM-CSF, TNFα, FLT3L, SCF, and either TGF-β1 or BMP7. After 7 days, pure LC fractions were obtained by CD207 positive magnetic

sorting. (A) Scheme depicting the *in vitro* differentiation procedure and flow cytometry plots showing the expression of the selected markers following magnetic sorting. (B) Representative flow cytometry plots (left) and graph (right) showing the mean fluorescence intensity (MFI) of PD-L1. (C) Representative flow cytometry plots (left) and graph (right) showing the MFI of PD-L2. Data are shown as mean ± SEM (n = 4). Data were analyzed using two-tailed paired Student's t-test. *P<0.05. Figure published in (Sconocchia *et al.*, 2020).

To continue and complement this analysis of BMP7-LCs, we differentiated TGF-β1-LCs and BMP7-LCs and sorted them for CD207 expression by magnetic sorting to obtain pure fractions (Figure 5A). Interestingly, we previously showed that these two LC types do not differ in the expression of activation markers (CD86 and HLA-DR) (Borek *et al.*, 2020). The extension of this analysis to also co-inhibitory molecules, revealed that TGF-β1-LCs and BMP7-LCs express different levels of PD-L molecules (Figure 5B). BMP7-LCs express slightly lower levels of PD-L1. However, interestingly, BMP7-LCs express higher levels of PD-L2, with a subset of these cells being PD-L2$^+$ (Figure 5C). Taken together these data suggest that TGF-β1-LCs and BMP7-LCs are functionally different.

4.3. BMP7-LCs are stronger than TGF-β1-LCs in promoting Tregs from naïve CD4$^+$CD45RA$^+$ T cells

DCs act as a bridge between innate and adaptive immunity. An important function of DCs, including LCs, is to interact with T cells to initiate/regulate an immune response. LCs play an important role in maintaining immune homeostasis by inducing Treg cells (Seneschal *et al.*, 2012; Kitashima *et al.*, 2018). In order to compare the functionality of TGF-β1-LCs and BMP7-LCs, enriched LC fractions were co-cultured with allogeneic naïve CD4$^+$CD45RA$^+$ T cells. Interestingly, after 5 days of culture with CD4$^+$ naïve T cells, significantly higher percentages of Tregs could be found in the BMP7-LC co-cultures in comparison with TGF-β1-LC co-cultures (Figure 6A).

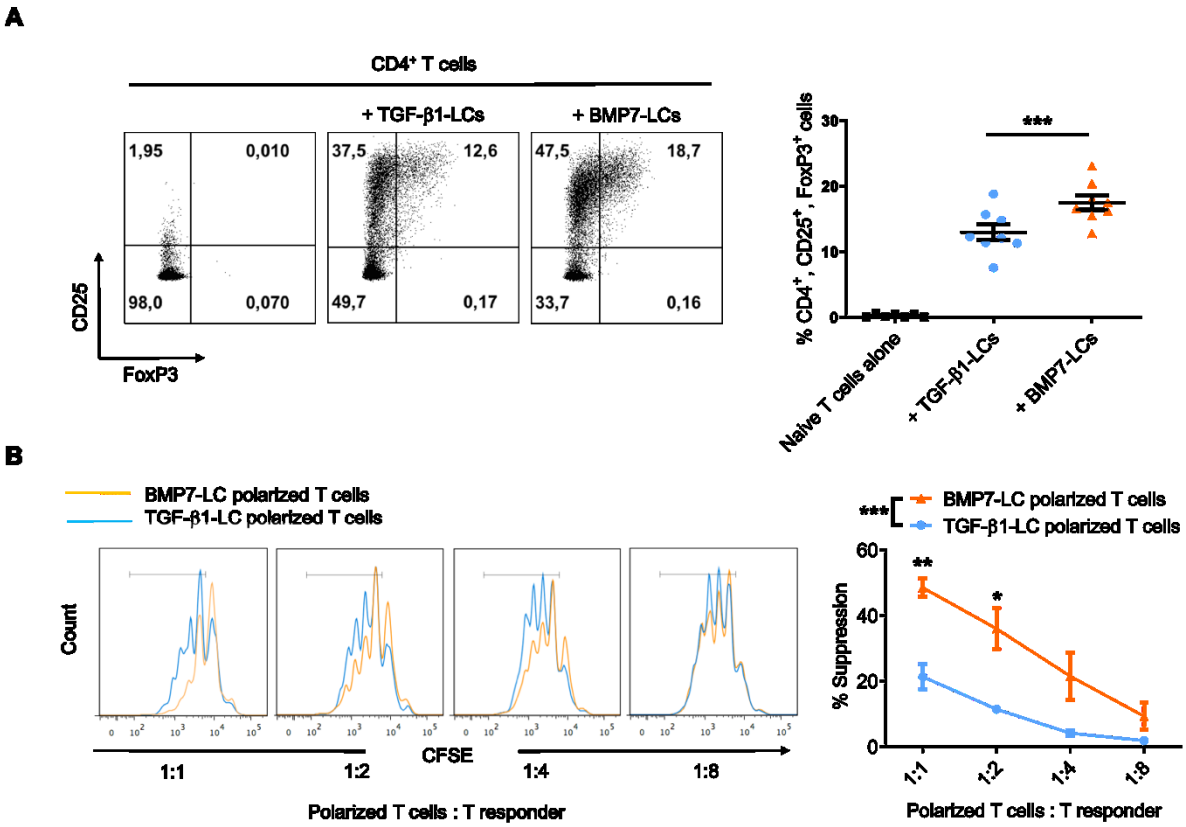

Figure 6. BMP7 are strong inducers of Treg differentiation from naïve CD4⁺ T cells. Enriched TGF-β1-LC and BMP7-LC fractions were cultured in the presence of allogeneic CD4⁺CD45RA⁺ naïve T cells for 5 days and then analyzed by flow cytometry for the presence of CD4⁺CD25⁺FoxP3⁺ Tregs. (A) Representative flow cytometry plots comparing CD25 and FoxP3 expression from CD4⁺ gated cells (left) and graph (right)showing the percentages of CD4⁺CD25⁺FoxP3⁺ Tregs. Data are shown as mean ± SEM (n = 8) and were analyzed by One-way ANOVA followed by Tukey's multiple comparison test. ***P<0.001. (B) Representative flow cytometry plots showing CFSE dilution in T responder cells after 4 days of co-culture with polarized T cells under different polarized T cell and T responder cell ratios (left). Graph (right) showing the percentage of suppression of T responder proliferation. Data are shown as mean ± SEM (n = two independent experiments performed in triplicates) and were analyzed using Two-way ANOVA followed by Sidak's multiple comparison test. *P<0.05, **P<0.01. Figure published in (Sconocchia *et al.*, 2020).

The functionality of the polarized T cells was then tested in a suppression assay by culturing increasing ratios of polarized T cells with and fixed amount of autologous CD4⁺ T

responder cells, stimulated with anti-CD3/CD28 beads. Indeed, T cells that were cultured with the BMP7-LCs exhibited a stronger ability to suppress T responder cell proliferation in comparison to those T cells cultured in the presence of TGF-β1-LCs (Figure 6B). This confirms that the BMP7-LCs are stronger at promoting Treg differentiation and that the Tregs are functionally active.

4.4. T cells cultured in the presence of BMP7-LCs secrete lower amounts of IL-22

In order to fully comprehend how TGF-β1-LCs and BMP7-LCs differ in their ability to induce Th cell polarization, we additionally analyzed by intracellular flow cytometry various cytokines that are secreted by the main Th cell subsets. These included IFNγ for Th1 cells, IL-4 for Th2 cells, IL-17A for Th17 cells, and IL-22 for Th22 cells. No significant differences were measured for IFNγ, IL-4, and IL-17A (Figure 7A and B). Interestingly, in the BMP7-LC co-cultures, less cells stained positive for IL-22 (Figure 7A and B), in comparison to the TGF-β1-LC co-cultures, suggesting that in the BMP7-LC condition less T cells produce IL-22. The intracellular flow cytometry measurements were confirmed by measuring the concentrations of the cytokines in the supernatants by Luminex. No significant differences were detected for IFNγ and IL-4 between the two conditions(Figure 7C; (Borek *et al.*, 2020)) corroborating what was observed with the intracellular flow cytometry data. We additionally confirmed that also in the supernatants, there are no differences in the concentrations of IL-17A between the two conditions and lower amounts of IL-22 in the BMP7-LC- T cell co-cultures (Figure 7C).

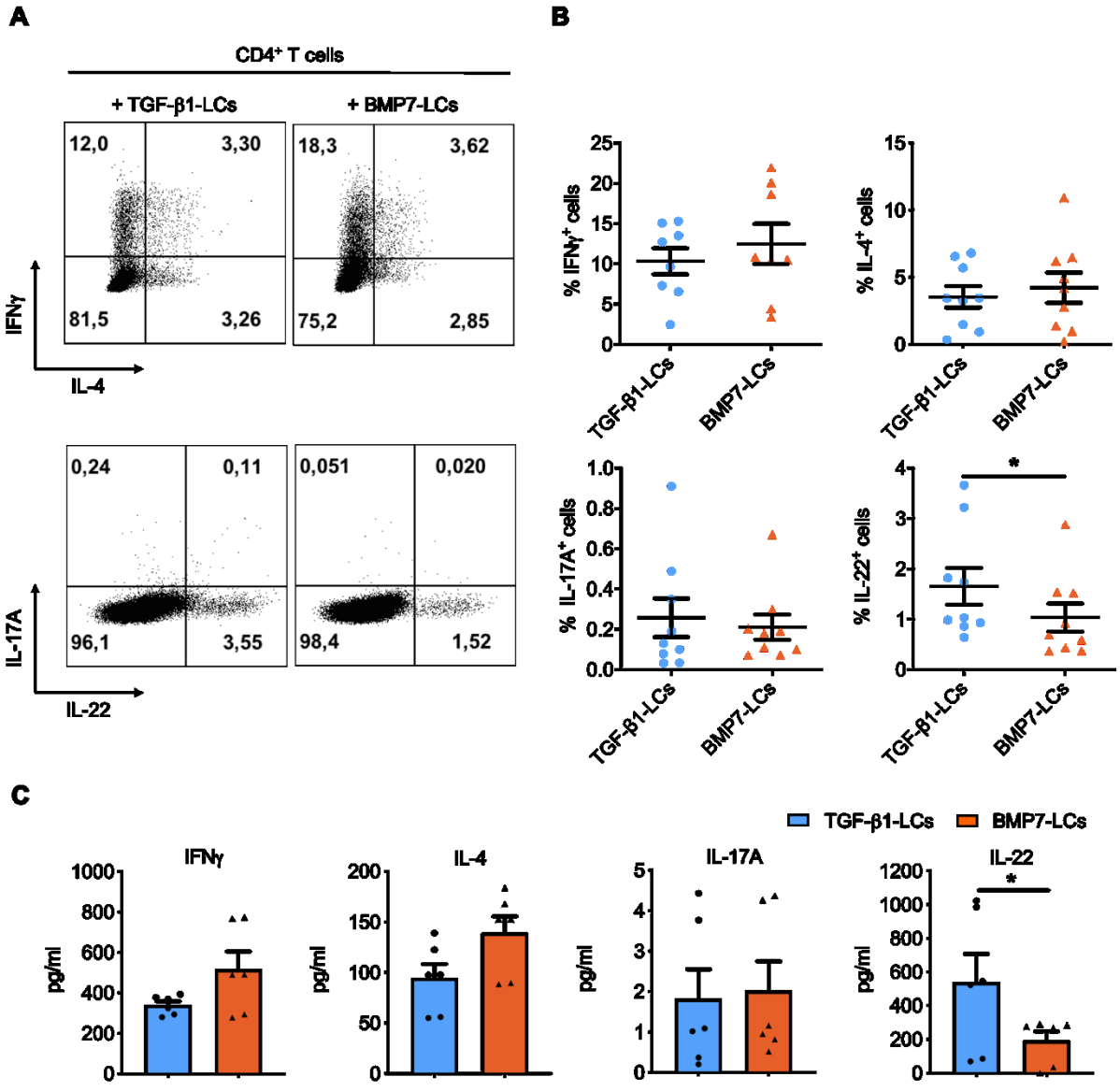

Figure 7. BMP7-LC primed T cells secrete lower amounts of IL-22 in comparison to TGF-β1-LC primed T cells. Enriched TGF-β1-LCs and BMP7-LCs were cultured in the presence of naïve CD4$^+$ T cells. After 5 days, T cells were pulsed for 4h with PMA/ionomycin in the presence on brefeldin A and cytokines were measured via intracellular flow cytometry. (A) Representative flow cytometry plots and (B) graphs showing percentages cells expressing IFNγ, IL-4, IL-17A, and IL-22 (n = 8-9). (C) Graphs showing the concentrations of the indicated cytokines in the supernatants after 5 days of culture (n = 3 independent experiments performed in duplicates). Data are shown as mean ± SEM and were analyzed using two-tailed Student's t-test. *P<0.05. Figure published in (Sconocchia *et al.*, 2020).

4.5. BMPs are secreted during LC-T cell co-cultures.

TGF-β1 is the most well-studied cytokine able to promote Treg differentiation (Chen *et al.*, 2003; Chen and Ten Dijke, 2016). This cytokine belongs to the bigger TGF-β family which is comprised of numerous members including BMPs and activins. Later studies showed that some of the other members of this family can also promote Treg differentiation (Semitekolou *et al.*, 2009). Therefore, we screened in the supernatants of the LC-T cell co-cultures for these proteins. From the series of proteins tested, TGF-β1, BMP2, and BMP7 were detected. No significant differences were detected in the concentrations of TGF-β1 between the two conditions. BMP2 was also detected, however, the concentrations were very low with no significant differences. Interestingly, BMP7 was only present in the supernatants of the BMP7-LC-T cell co-cultures (Figure 8A).

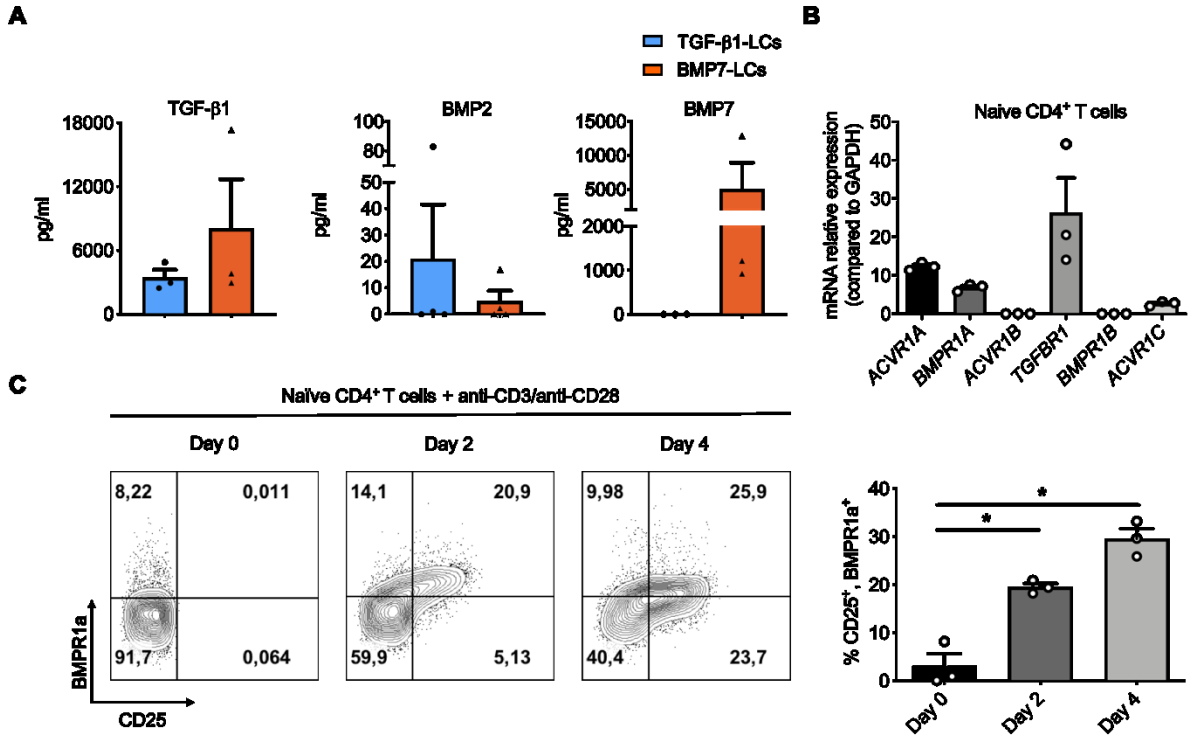

Figure 8. Activated naïve CD4⁺ T cells can respond to secreted BMP ligands. Supernatants were collected after 3 days of culturing TGF-β1-LCs or BMP7-LCs with naïve CD4⁺ T cells and TGF-β family cytokines were measured. (A) Graphs showing the concentrations of TGF-β1, BMP2, and BMP7. Data are shown as mean ± SEM (n = 3). (B) Graph showing the relative mRNA expression measured by qPCR of the different TGF-β family type I receptors in resting naïve CD4⁺ T cells. GAPDH was used as housekeeping gene. Data are shown as mean ± SEM (n = 3). (C) Representative flow cytometry plots (left) and graph

(right) showing the percentages of expression of BMPR1a and CD25 on naïve $CD4^+$ T cells following stimulation with immobilized anti-CD3 (3 μg/mL) and soluble anti-CD25 (1 μg/mL) at days 0, 2, and 4. Data are shown as mean ± SEM (n = 3) and were analyzed by One-way ANOVA followed by Tukey's multiple comparison test. *P<0.05. Figure published in (Sconocchia *et al.*, 2020).

It was previously described that T cells are equipped with the machinery to respond to BMP ligands and that BMP signaling is involved in T cell homeostasis, function, and differentiation (Hager-Theodorides *et al.*, 2002; Martínez *et al.*, 2015). We confirmed this observation by showing that freshly isolated naïve $CD4^+$ T cells express mRNA for different TGF-β type I receptors. The highest expressed receptor is TGFβR1, followed by ACVR1A, and BMPR1A. Very low mRNA expression was detected for ACVR1C and no expression was detected for ACVR1B and BMPR1B (Figure 8B). At a protein level, BMPR1a is poorly expressed on naïve $CD4^+$ T cells. Following stimulation of the TCR and co-stimulation with anti-CD28, T cells upregulate BMPR1a together with the IL-2 receptor CD25 (Figure 8C). These data suggest that following activation, T cells are able to respond to paracrine/autocrine BMPs that are available in the micro-environment.

4.6. BMPR1a signaling is involved in BMP7-LC-mediated Treg differentiation

To understand the role of BMPR1a signaling in the LC-T cell co-cultures, we inhibited BMPR1a signaling by culturing TGF-β1-LCs and BMP7-LCs with a soluble BMPR1a-Fc chimera protein. Regarding $CD4^+CD25^+FoxP3^+$ Treg differentiation, the addition of the BMPR1a inhibitor had no significant effect in the TGF-β1-LC co-cultures. Interestingly, when added to the BMP7-LC condition, where BMP7 was detected in the supernatants, the BMPR1a inhibitor caused a significant decrease in the percentages of Tregs found in the cultures after 5 days (Figure 9A). The addition of the BMPR1a inhibitor had no significant effects regarding the percentages of cytokine expression, with no significant differences in the percentages of T cells that expressed IFNγ, IL-4 (Figure 9B), IL-17A, and IL-22 (Figure 9C). We also extended our analysis to the anti-inflammatory cytokine IL-10 but found no significant differences (Figure 9D). These data suggest that secreted BMP ligands can contribute to the differentiation of Tregs through BMPR1a.

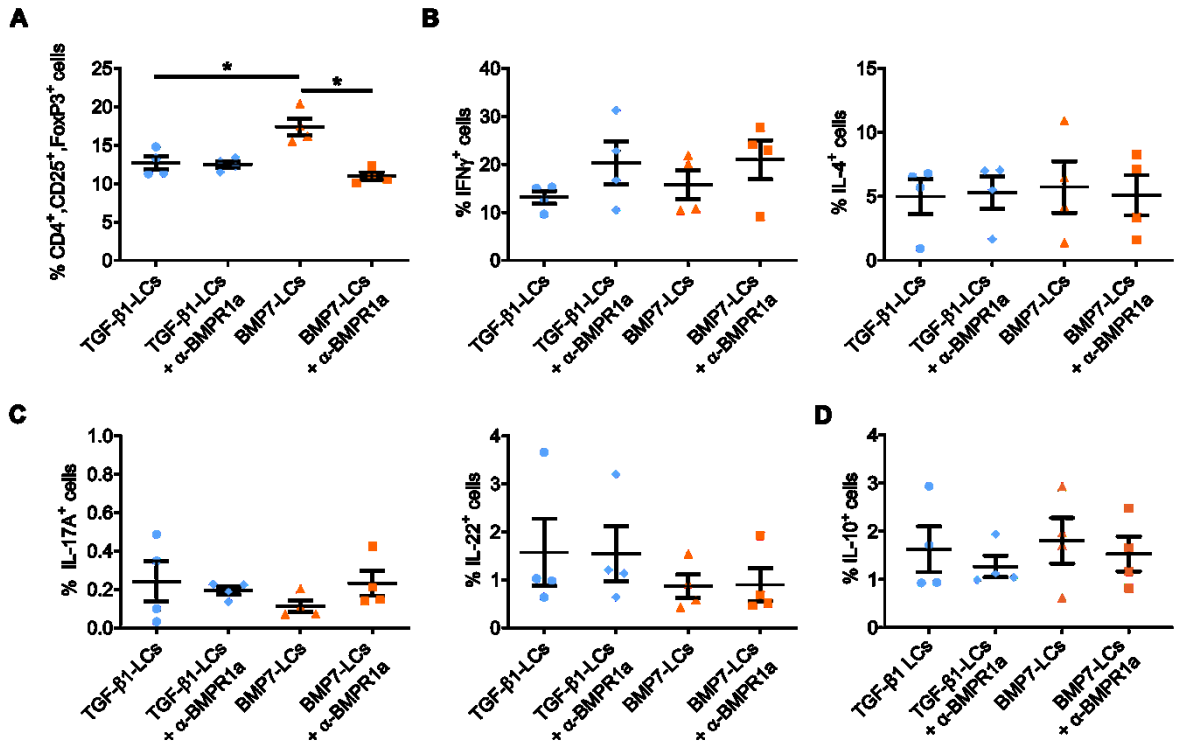

Figure 9. Inhibition of BMPR1a signaling inhibits partially Treg induction by BMP7-LCs. TGF-β1-LCs or BMP7-LCs were cultured in the presence of allogeneic naïve $CD4^+$ T cells. After 5 days, cells were analyzed for Tregs and intracellular cytokines by flow cytometry. For intracellular cytokine detection, cells were pulsed with PMA/ionomycin in the presence of brefeldin A for 4h before measuring. (A) Graph showing percentages of $CD4^+CD25^+FoxP3^+$Tregs. (B) Graphs showing percentages of IFNγ and IL-4 positive cells. (C) Graphs showing percentages of IL-17A and IL-22 positive cells. (D) Graph showing percentages of IL-10 positive cells. Data are shown as mean ± SEM (n = 4). Data were analyzed using One-way ANOVA followed by Tukey's multiple comparison test. *P<0.05. Figure published in (Sconocchia *et al.*, 2020).

4.7. Direct addition of BMP7 promotes Treg differentiation

To further provide evidence that BMP7 and BMPR1a signaling can directly promote Treg differentiation in naïve $CD4^+$ T cells, we tested whether exogenous BMP7 added to naïve $CD4^+$ T cells, in the absence of APCs, could promote Tregs. Standard protocols used for the differentiation *in vitro* of $FoxP3^+$ Tregs rely on the treatment of naïve $CD4^+$ T cells with IL-2 and TGF-β1 in the presence of TCR stimulation. We compared whether substitution of TGF-β1 with BMP7 in this protocol could also promote the generation of Tregs. As expected, the standard

protocol containing TGF-β1 gave rise to large percentages of Tregs. Interestingly, the replacement of TGF-β1 with BMP7 was also able to promote Tregs, although not as efficiently as TGF-β1. Nevertheless, BMP7 was able to double the percentages of Tregs in culture in comparison to the media control. Furthermore, the simultaneous addition of TGF-β1 and BMP7 led to a small but significant additional effect (Figure 10A). On the other hand, the percentages of IFNγ$^+$ T cells were inversely correlated to the percentages of FoxP3$^+$ Tregs, with significantly lower expression of IFNγ in the T cells exposed to TGF-β1 and BMP7 in comparison to the media controls (Figure 10B).

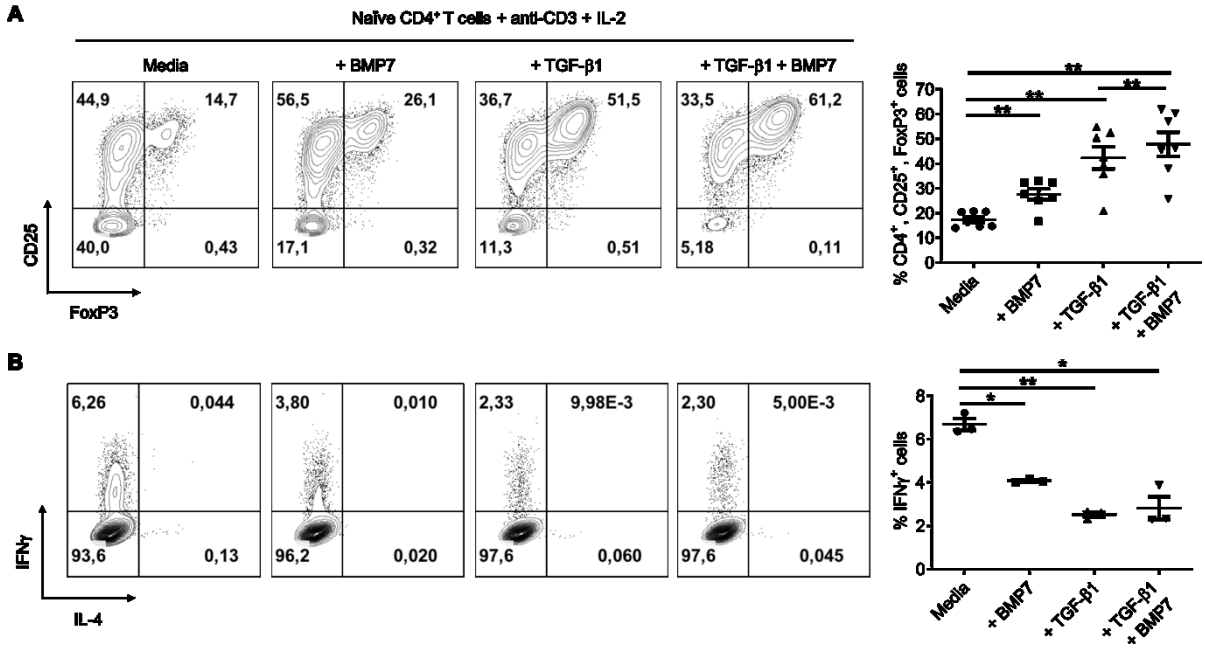

Figure 10. BMP7 directly promotes Treg differentiation. Naive CD4$^+$ T cells were cultured in serum-free conditions in the presence of 3 μg/mL immobilized anti-CD3, 50 ng/mL of IL-2, and 3 ng/mL of TGF-β1 to promote Treg differentiation. 200 ng/mL of BMP7 was either used to replace TGF-β1 or added together with TGF-β1. The culture media with anti-CD3 and IL-2 was used as the media control. After 4 days, T cells were analyzed for Tregs and for intracellular IFNγ. For intracellular cytokine detection, cells were first pulsed for 4h with PMA/ionomycin and brefeldin A. (A) Representative flow cytometry plots (left) showing the expression of CD25 and FoxP3 on gated CD4$^+$ T cells and graphs (right) showing the percentages of CD4$^+$CD25$^+$FoxP3$^+$ Tregs (n = 7). (B) Representative flow cytometry plots (left) showing the expression of intracellular IFNγ and IL-4 and graph (right) showing the

percentages of IFNγ-positive cells (n = 3). Data are shown as mean ± SEM and were analyzed by One-way ANOVA followed by Tukey's multiple comparison test. *P<0.05, **P<0.01. Figure published in (Sconocchia *et al.*, 2020).

4.8. BMP signaling promotes Treg differentiation by promoting CD25 expression

In addition to stably expressing FoxP3, another important marker for Tregs is that they express constitutively high levels of the IL-2 receptor CD25. This receptor binds IL-2 which plays an important role for Treg maintenance, by stabilizing FoxP3 expression (Fontenot *et al.*, 2005). Moreover, the high expression of CD25 on Tregs also plays a functional role because by expressing higher levels of this receptor they are able to sequester IL-2 from effector T cells (Chinen *et al.*, 2016). BMP7 induced CD25 expression in a similar manner as TGF-β1 (Figure 11A). To better understand how BMP signaling contributes to Treg differentiation we repeated the study in the presence of the TGFβR1 inhibitor, SB431542 (SB), or in the presence of dorsomorphin (DM) which inhibits BMP signaling. As expected, the addition of SB to the TGF-β1 cultures resulted in the reduction of $CD25^{+}FoxP3^{+}$ Tregs and no significant effects on the percentages of Tregs in the cultures where BMP7 was added (Figure 11B). Strikingly, the addition of DM not only inhibited Treg differentiation in the BMP7 condition but also inhibited it in the TGF-β1 condition (Figure 11B). To further investigate this effect seen with DM, we divided the cells into $CD25^{+}FoxP3^{+}$ and $CD25^{+}FoxP3^{-}$ cells. The addition of SB to the TGF-β1 cultures led to a decrease in the ratio between the two groups, whereas the addition of DM, even though it also caused a decrease in Treg percentages, failed to modify this ratio (Figure 11C). What could be observed however, was a striking decrease in CD25 expression (Figure 11D).

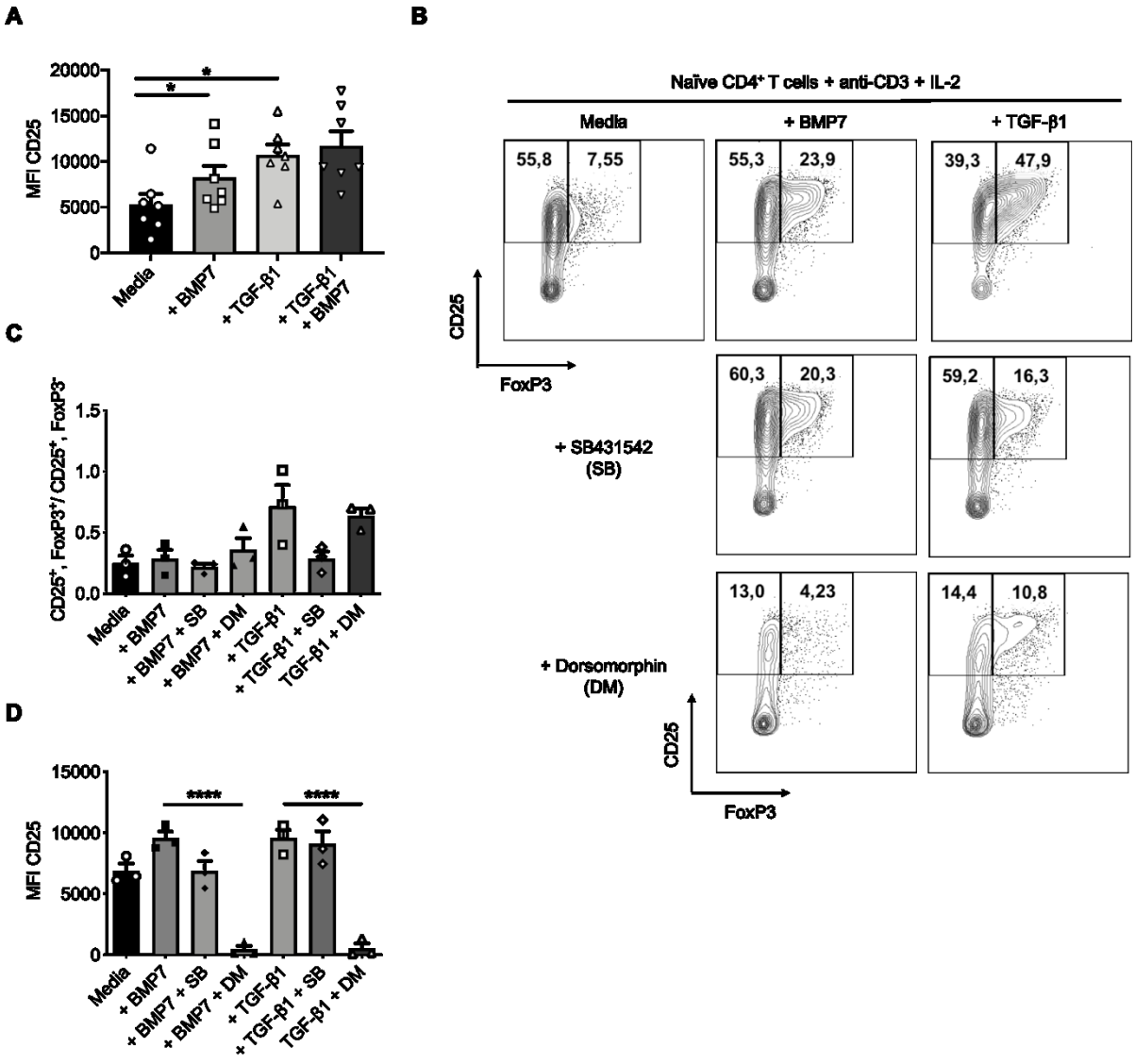

Figure 11. BMP signaling regulates CD25 expression. Naïve $CD4^+$ T cells were cultured in serum-free conditions in the presence 3 μg/mL immobilized anti-CD3, 50 ng/mL IL-2, and 3 ng/mL TGF-β1 or 200 ng/mL BMP7 or both for 4 days. Where indicated, 5 μM of either SB431542 (SB), to inhibit TGF-β signaling, or dorsomorphin (DM), to inhibit BMP signaling, were added. (A) Graph showing CD25 MFI after 4 days of culture in the indicated conditions (n = 7). (B) Representative flow cytometry plots showing the expression of CD25 and FoxP3 (n = 3) on pre-gated $CD4^+$ T cells. (C) Graph showing the ratio between $CD25^+FoxP3^+$ and $CD25^+FoxP3^-$ $CD4^+$ T cells (n = 3). (D) Graph showing CD25 MFI (n = 3) and (E) intracellular IL-2 expression (n = 3) after 4 days of culture under the indicated conditions. Data are shown as mean ± SEM and were analyzed using One-way ANOVA followed by Tukey's multiple

comparison test. *P<0.05, ***P<0.001, ****P<0.0001. Figure published in (Sconocchia *et al.*, 2020).

BMP was previously described to modulate IL-2 expression in T cells. Therefore, we also measured IL-2 in expression the various experimental conditions. Interference with the BMP signaling, through the treatment of the cells with DM, caused a strong significant decrease in IL-2 production (Figure 12).

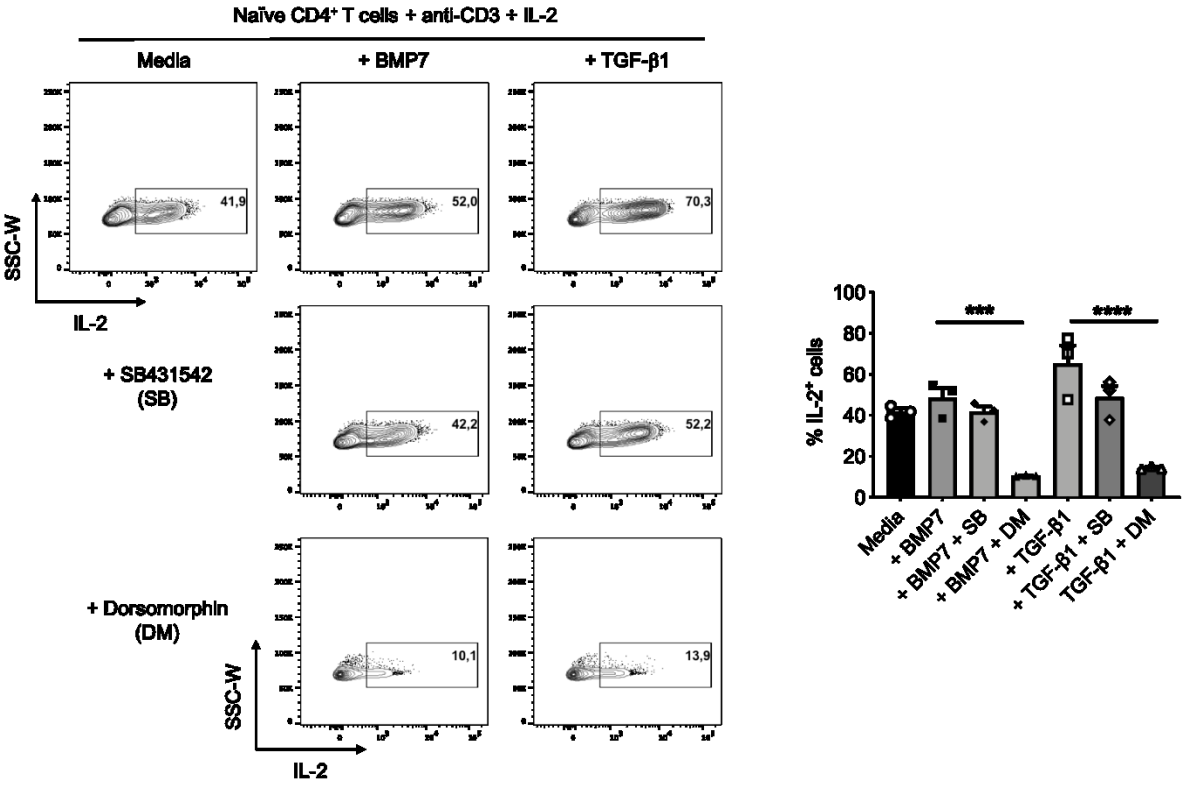

Figura 12. BMP signaling regulates IL-2 production in CD4⁺ T cells. Naïve CD4⁺ T cells were cultured in serum-free conditions in the presence 3 μg/mL immobilized anti-CD3, 50 ng/mL IL-2, and 3 ng/mL TGF-β1 or 200 ng/mL BMP7 or both for 4 days. Where indicated, 5 μM of either SB431542 (SB), to inhibit TGF-β signaling, or dorsomorphin (DM), to inhibit BMP signaling, were added. Representative flow cytometry plots showing the expression of intracellular IL-2 following re-stimulation of the cells with PMA/Ionomycin in the presence of brefeldin A for 4h (left). Graphs showing percentages of IL-2⁺ T cells (right, n=3). Data are shown as mean ± SEM and were analyzed using One-way ANOVA followed by Tukey's multiple comparison test. ***P<0.001. Figure published in (Sconocchia *et al.*, 2020).

Taken together, these data suggest that the effect of DM on the reduction of FoxP3 expression is an indirect effect. These data propose that BMP signaling is important for the expression of CD25 and IL-2, two elements that play an important role in the subsequent expression of FoxP3 during TGF-β1- or BMP7-mediated Treg differentiation from naïve $CD4^+$ T cells.

4.9. Tregs in psoriatic skin lesions display active BMP downstream signaling

Based on the previously described results that during psoriasis BMP7 expression is strongly enhanced and that BMP signaling is involved in Treg differentiation, we analyzed skin biopsies from healthy donors and from lesions from psoriatic patients for the activation of BMP signaling. We detected strong activation of the BMP downstream signaling component pSMAD1/5/8 in the psoriatic lesions, with 31.9% ± 6.1% of $FoxP3^+$ cells that also expressed pSMAD1/5/8 (Figure 12A and B). In contrast, no $FoxP3^+$ cells in the healthy skin showed activation of the BMP signaling pathway (Figure 12A and B).

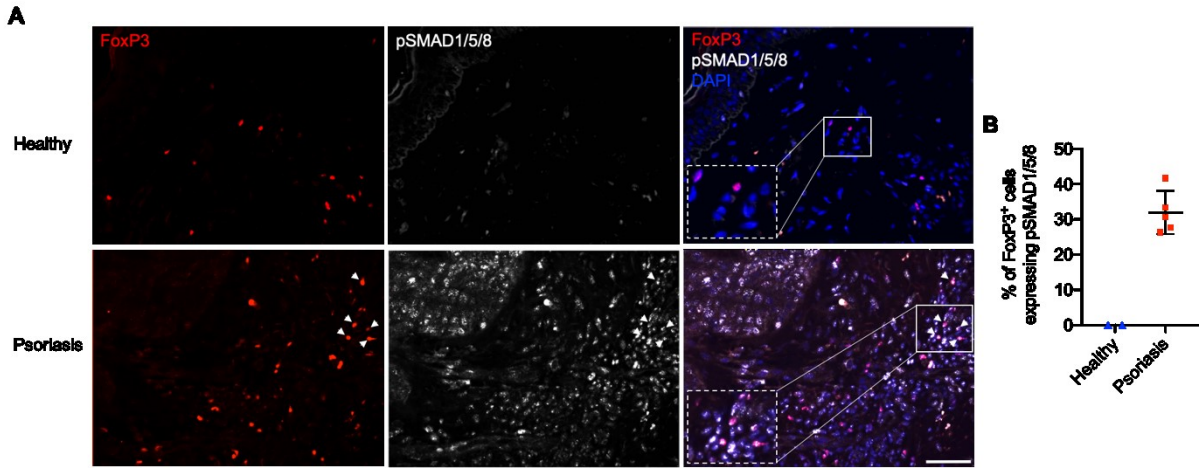

Figure 13. Active BMP signaling is present in $FoxP3^+$ cells in psoriatic skin lesions. (A) Representative immunofluorescence stainings of sections from lesional psoriatic skin (n = 6) and healthy skin (n = 2) analyzed for the expression of pSMAD1/5/8 (white) and FoxP3 (red). DAPI was used to visualize the nuclei. The scale bar represents 50 μm. (B) Graph showing the percentages of $FoxP3^+$ cells expressing pSMAD1/5/8 (n = 2-6). Data are shown as mean ± SD. Figure published in (Sconocchia *et al.*, 2020).

4.10. Short-term BMP7 stimulation does not influence moDC lineage marker expression but promotes PD-L1/2 expression

During inflammation, monocytes from the bloodstream can reach the inflamed tissues where they can assume DC features. BMP7 was previously described to promote the expression of LC features (Milne *et al.*, 2015). Therefore, we tested whether BMP7 could also induce LC-characteristics in differentiated moDCs. Treatment of moDCs with BMP7 for 48h led to no significant changes in the expression of CD1a and CD11b (Figure 13, top). Additionally, it did not promote the expression of CD207 and CD324/E-Cadherin, which are typical markers expressed on LCs (Figure 13, bottom).

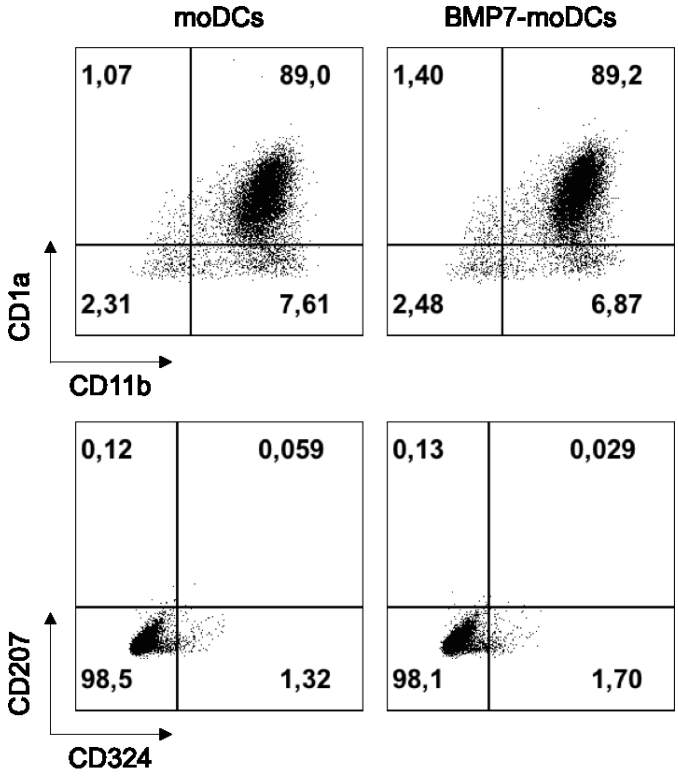

Figure 14. BMP7 stimulated moDCs do not express LC markers. MoDCs were differentiated from CD14^{+} monocytes with 100 ng/mL of GM-CSF and 35 ng/mL of IL-4 for 6 days. Afterwards, differentiated moDCs were treated for 48h with 200 ng/mL of BMP7 and analyzed by flow cytometry. Flow cytometry plots showing the expression of CD1a and CD11b (top) and CD207 and CD324/E-Cadherin (bottom). The figure shows the results of a

representative experiment chosen from three independent experiments that were performed with similar results. Figure published in (Sconocchia *et al.*, 2020).

Next, we extended our study to investigate whether BMP7 could influence the expression of co-stimulatory and co-inhibitory markers. Short-term treatment of moDCs with BMP7 slightly increased the expression of the maturation markers HLA-DR, CD80, and CD86. However, the effect was not consistent and failed to reach statistical significance. Interestingly, BMP7-primed moDCs expressed significantly higher PD-L1 and PD-L2 (Figure 14B). Taken together, these data indicate that stimulation of moDCs with BMP7 promotes the expression of co-inhibitory molecules without altering the expression of moDC lineage markers or promoting LC-characteristics.

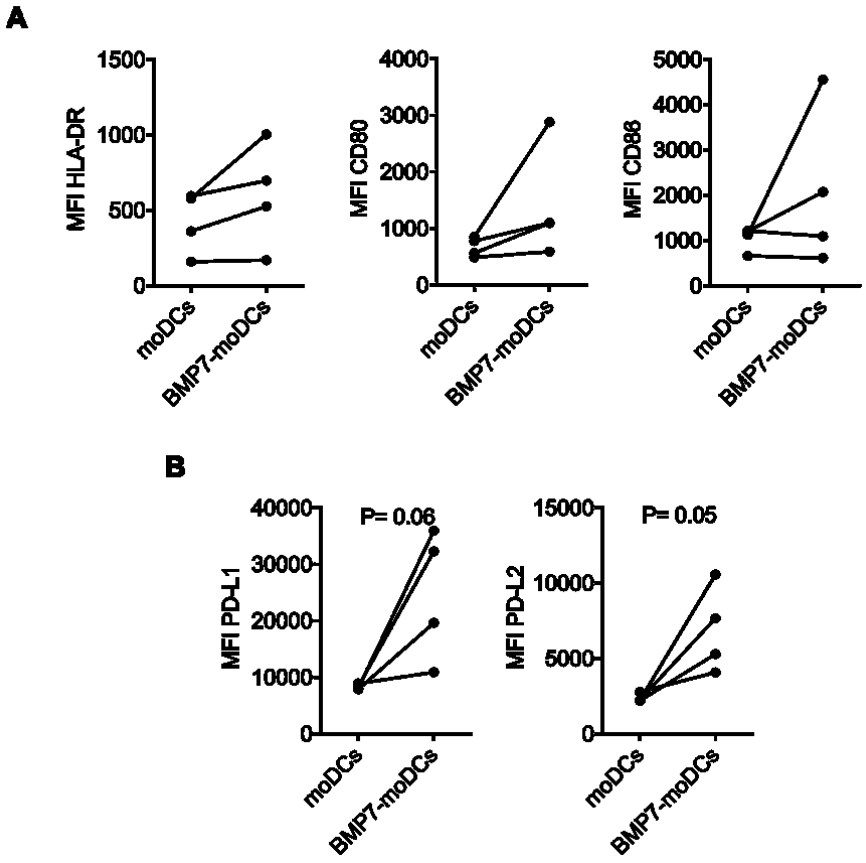

Figure 15. BMP7 promotes PD-L1/2 expression in moDCs. MoDCs, differentiated from $CD14^+$ monocytes with 100 ng/mL of GM-CSF and 35 ng/mL of IL-4, were treated for 48h with 200 ng/mL of BMP7 and analyzed by flow cytometry. (A) Graphs showing the MFIs of HLA-

DR, CD80, and CD86 in moDCs (n = 4). (B) Graphs showing the expression of PD-L1 and PD-L2 in moDCs (n = 4). Data are shown as mean ± SEM and were analyzed using paired two-tailed Student's t-test. Figure published in (Sconocchia *et al.*, 2020).

4.11. BMP7-primed moDCs display enhanced FoxP3$^+$ Treg promoting ability

To functionally test the effects of the short-term treatment of moDCs with BMP7, moDCs were cultured in the presence of allogeneic naïve CD4$^+$CD45RA$^+$ T cells. To remove all the BMP7 that was added to stimulate the moDCs, the cells were first extensively washed and then cultured in fresh media with the T cells. Similar to what was observed with BMP7-LCs, BMP7 could be detected in the supernatants of the BMP7-moDC-T cell co-cultures (Figure 15A). Additionally, BMP7-moDCs were stronger in promoting FoxP3$^+$ Treg differentiation compared to untreated moDCs and the addition of a soluble BMPR1a inhibitor cancelled this effect (Figure 15B).

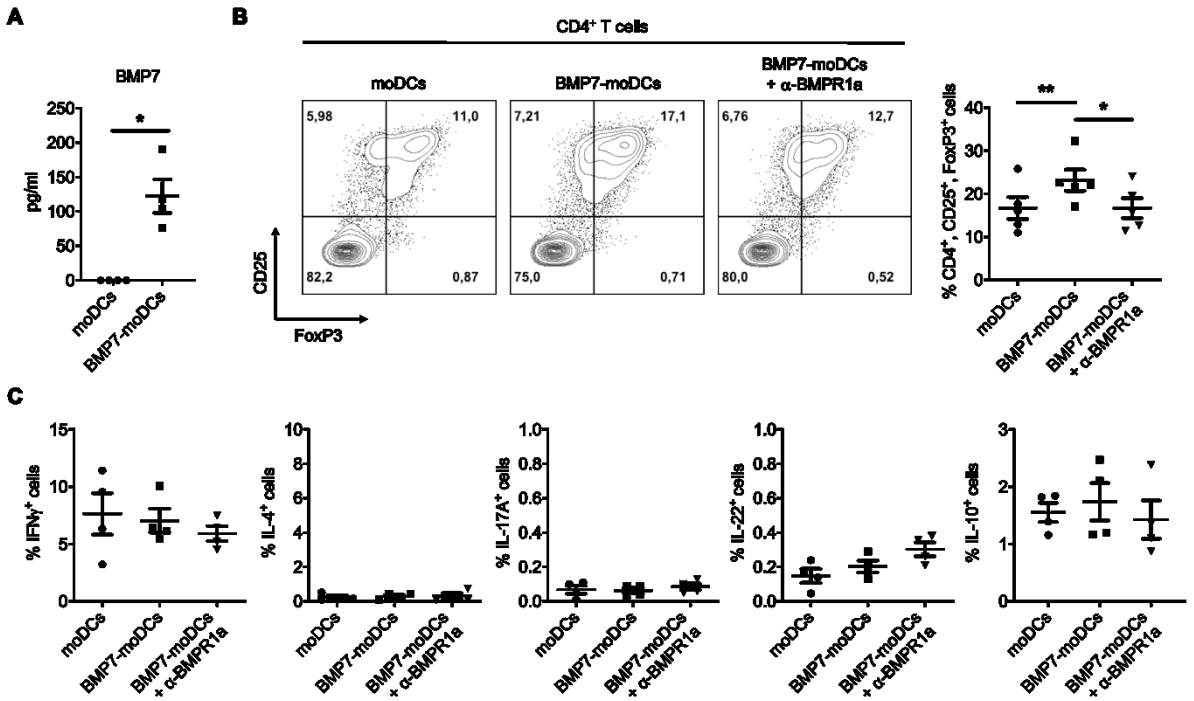

Figure 16. BMP7 stimulation of moDCs enhances their ability to promote Tregs. MoDCs, treated with or without BMP7, were cultured in the presence of allogeneic naïve CD4$^+$CD45RA$^+$

T cells. After 3 days, supernatants were collected for cytokine analysis and after 5 days, cells were analyzed by flow cytometry. For intracellular cytokine detection, T cells were first pulsed with PMA/ionomycin in the presence of brefeldin A for 4h. (A) Graphs shows the concentrations of BMP7 in the supernatants of MLRs after 3 days measured by ELISA (n = 4). Data are shown as mean ± SEM and were analyzed using two-tailed paired Student's t-test. (B) Representative flow cytometry plots showing the expression of CD25 and FoxP3 on gated $CD4^+$ T cells and graph showing percentages of $CD4^+CD25^+FoxP3^+$ Tregs (n = 5). (C) Graphs showing percentages of cells expressing IFNγ, IL-4, IL-17A, IL-22, and IL-10 (n = 4). Data are shown as mean ± SEM and were analyzed by One-way ANOVA followed by Tukey's multiple comparison test. *P<0.05, **P<0.01. (Sconocchia *et al.*, 2020).

Short-term stimulation of moDCs with BMP7 did not lead to striking or significant changes in terms of intracellular cytokine expression by the T cells. The most abundantly detected cytokine was IFNγ followed by IL-10, suggesting that moDCs mainly prime naïve T cells to a Th1 subset. The addition of a BMPR1a inhibitor slightly decreased IL-10 percentages but not sufficiently to reach statistical significance. Almost no IL-4 and IL-17A could be detected and the addition of a BMPR1a inhibitor did not influence their expression. Very low amounts of IL-22 were detected and the addition of a BMPR1a inhibitor slightly increased the percentages. However, this increase was considerably small and did not reach statistical significance (Figure 15C).

Taken together, these data suggest that short-term stimulation of moDCs with BMP7 enhances their ability to promote Treg differentiation in a BMPR1a-dependent manner.

4.12. Loss of BMPR1a signaling in $CD11c^+$ DCs results in increased psoriasis-like inflammation

To gain more *in vivo* relevance on the role of BMPR1a signaling in DCs, a conditional KO mouse was bred in which BMPR1a is specifically knocked out of cells expressing CD11c (referred to from now on as BMPR1aΔCD11c mice), thus including DCs and LCs. This mouse model was generated by breeding BMPR1a-loxP-flanked mice with CD11c-cre mice. Under steady-state conditions, BMPR1aΔCD11c mice displayed no differences in phenotype in comparison to the WT mice. To mimic psoriasis-like skin inflammation, the mice were treated topically on the ears with the TLR7 agonist imiquimod for 6 consecutive days (Figure 16A). During these days, the change in ear thickness, epidermal LC network, BMP7 expression and number of $FoxP3^+$ cells were monitored.

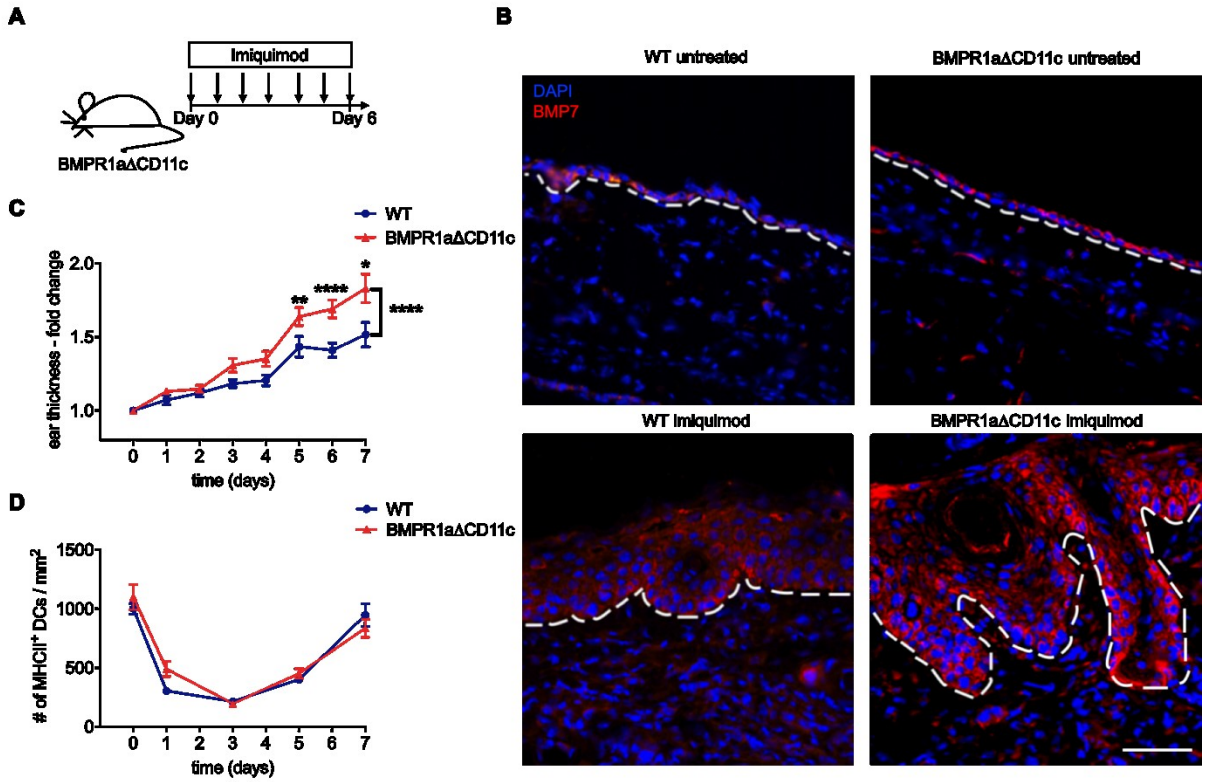

Figure 17. BMPR1aΔCD11c mice exhibit stronger skin inflammation after imiquimod treatment. (A) Representative scheme illustrating the time span of the imiquimod treatment. (B) Representative ear skin sections from WT and BMPR1aΔCD11c mice untreated (top) and after 6 days of imiquimod treatment (bottom) analyzed for the expression of BMP7 (red). The nuclei were visualized with DAPI and the white dotted line separates the dermis from the epidermis. The scale bar represents 50 μm. (C) Graph showing the fold change in ear thickness measured using a caliper following imiquimod treatment. The fold change was calculated by dividing the ear thickness of each mouse by its respective ear thickness on day 0 (n = 13-18). (D) Epidermal ear sheets were stained for MHCII and counted. Graph showing the number of LCs ($MHCII^+$ cells) per mm^2 at the indicated days (n = 4-13). Data are shown as mean ± SEM and were analyzed using Two-way ANOVA followed by Sidak's multiple comparison test. *P<0.05, **P<0.01, ****P<0.0001. Figure published in (Sconocchia *et al.*, 2020).

BMP7 expression pattern in the mouse skin was similar to what was seen in human skin. In the absence of inflammation, BMP7 has mainly a mono-layer expression confined to the suprabasal layer of the epidermis (Figure 16B, top). However, following skin inflammation, enhanced BMP7 expression is detected with its expression not being limited only to the suprabasal epidermal layer (Figure 16B, bottom). Daily topical treatment of the mice's ears with imiquimod led to increased ear swelling in both conditions, with the BMPR1aΔCD11c mice exhibiting significantly stronger ear swelling on days 5, 6, and 7 in comparison to the WT mice (Figure 16C).

Studies have demonstrated that LCs can play a protective role during psoriasis (Glitzner *et al.*, 2014). Since BMPR1a signaling has been described to be involved in LC differentiation (Yasmin *et al.*, 2013), to exclude that this higher inflammation might be due to an effect on LC differentiation, we tracked the LC network during the imiquimod treatment by quantifying the number of $MHCII^+$ cells present in the epidermis. Following treatment with imiquimod, the number of LCs decreased over time, with the lowest number of LCs at day 3, to then repopulate the epidermis by day 7 (Figure 16D). No significant differences were detected between WT and BMPR1aΔCD11c mice suggesting that this effect is not due to differences in LC frequencies in the epidermis.

We next calculated the number of $FoxP3^+$ cells present in the skin during the course of inflammation. Similar to what was seen in the human samples, there is accumulation of $FoxP3^+$ cells in the skin during psoriasis-like inflammation. No significant differences in Treg numbers was observed before the start of the treatment (day 0). Interestingly, at day 6, when the largest difference in ear thickness was observed between WT and BMPR1aΔCD11c mice, the BMPR1aΔCD11c mice also contained significantly less $FoxP3^+$ cells in the skin (Figure 17A and B).

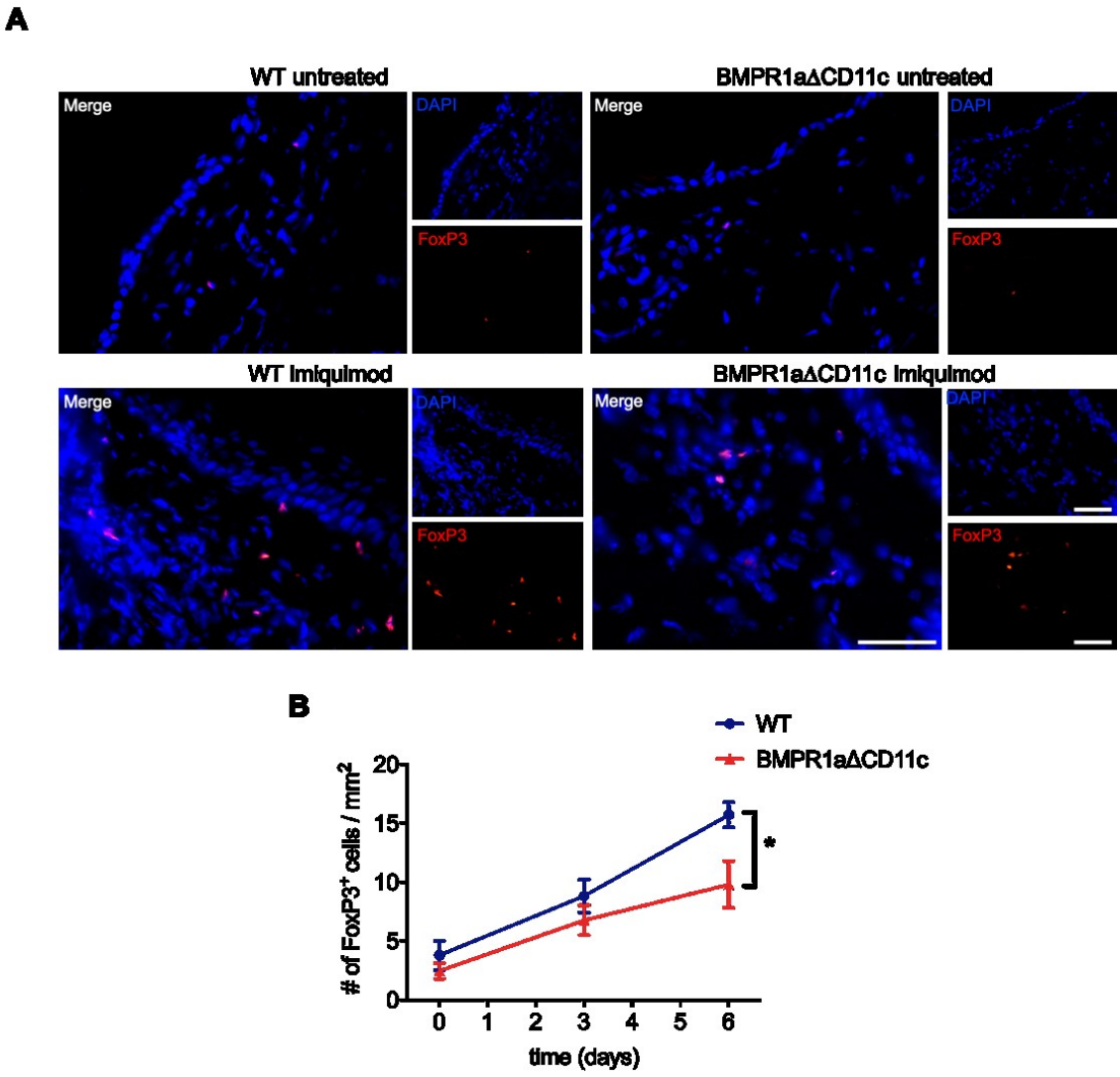

Figure 18. Lower FoxP3⁺ cells are present during psoriasis-like skin inflammation in BMPR1aΔCD11c mice. (A) Representative immunofluorescence stainings of ear skin sections untreated (top) and day 6 treated (bottom) WT and BMPR1aDCD11c mice analyzed for the expression of FoxP3 (red). The nuclei were visualized by DAPI staining. The scale bar represents 50 μm. (B) Graph showing the number of FoxP3⁺ cells per mm^2 at days 0, 3, and 6 of the experiment (n = 3-7). Data are shown as mean ± SEM and were analyzed using Two-way ANOVA followed by Sidak's multiple comparison test. *P<0.05. Figure published in (Sconocchia *et al.*, 2020).

5. Discussion

LCs are unique epidermal DCs particularly known for their ability to maintain immune homeostasis during steady-state by promoting Treg differentiation from naïve $CD4^+$ T cells and proliferation of skin resident $CD4^+CD45RO^+$ Tregs (Seneschal *et al.*, 2012; Kitashima *et al.*, 2018). During inflammation, Tregs accumulate in the affected tissues and regulate the inflammatory process (Hirahara *et al.*, 2006). Treg depletion studies in mice confirmed that Tregs play an active role in psoriasis-like skin inflammation by limiting the aggravation of the condition (Hartwig *et al.*, 2018; Stockenhuber *et al.*, 2018). However, the underlying mechanisms have not been so far fully elucidated. We here showed that BMP receptor signaling is involved in the regulation of skin inflammation by modulating the function of DCs/LCs and T cell subset differentiation. We described that in lesions from psoriatic skin BMP7 is strongly overexpressed with an expression pattern that differs from that observed in healthy controls and that BMP7 promotes the generation of LCs that phenotypically resemble a subset of LCs found in psoriatic skin lesions. In addition, the detection of active BMP downstream signaling indicated that various cellular components of the skin respond to the BMP7. We here demonstrated that the amount of $FoxP3^+$ Tregs positively correlates with the expression intensity of BMP7 within the lesional epidermis both in psoriatic patients and in imiquimod-induced murine psoriasis-like inflammation. Additionally, we depict a functional connection between Treg accumulation and increased BMP signaling. In more detail, we describe that BMP signaling instructs LCs/moDCs to more strongly promote Treg differentiation from naïve $CD4^+$ T cells. The addition of a BMPR1a/ALK3 inhibitor blocked this effect, indicating that this effect is mediated by ligands of such receptor like BMP7 that are released during the interaction between LCs/moDCs and naïve $CD4^+$ T cells. To further support these observations, the treatment of naïve $CD4^+$ T cells with BMPR1a ligands promotes the differentiation of Tregs, through a mechanism that involves the upregulation of the IL-2 receptor CD25 and that is distinct from the classical TGF-β1 pathway, which is the most commonly associated cytokine involved in *in vitro* Treg differentiation. Our data postulate a model in which cognate DC-mediated T cell receptor activation leads to the upregulation of BMPR1a in naïve $CD4^+$ T cells, enabling these cells to be exposed to strong lesional BMP7-BMPR1a signaling, in turn boosting Treg generation. Therefore, aberrant high BMP signaling in the psoriatic lesional skin instructs inflammatory LC/DCs to gain enhanced Treg stimulatory activity, and locally secreted TGF-β family ligands directly promote Tregs through the BMP signaling cascade.

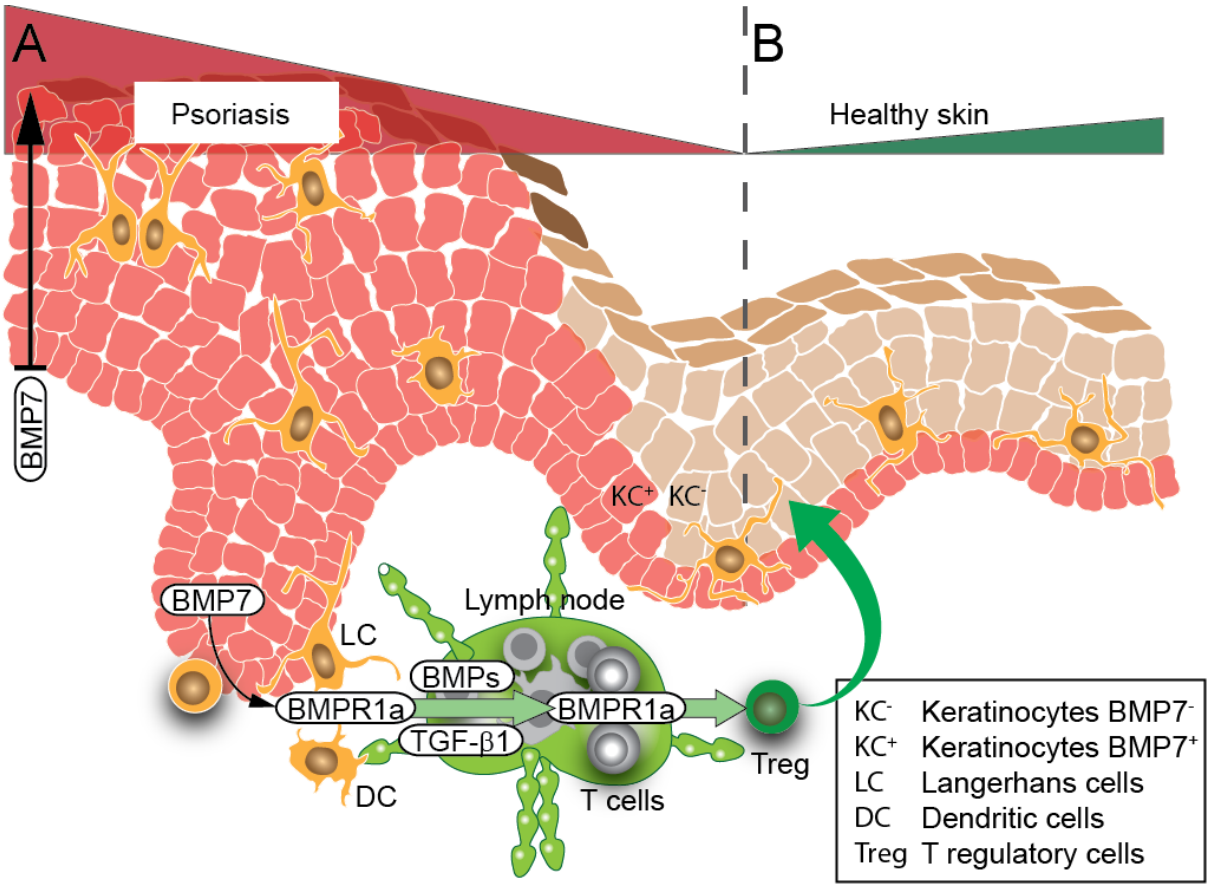

Figure 19. BMP receptor-mediated Treg expansion by dendritic cells. Proposed model of Treg expansion by dendritic cells mediated through a BMPR1a-dependant mechanism. During psoriasis, aberrantly high levels of BMP7 can promote differentiation of LCs and act upon infiltrating moDCs. BMP7-exposed cells secrete TGF-β1 ligands (TGF-β1, BMP2, BMP7) that can promote the differentiation of naïve $CD4^+$ T cells into $FoxP3^+$ Tregs. Figure published in (Sconocchia *et al.*, 2020).

The role of LCs in psoriasis has not fully been elucidated. However, recent studies performed by depleting LCs in murine models of psoriasis-like skin inflammation have exposed that LCs exercise an attenuating role in psoriasis-like skin inflammation (Glitzner *et al.*, 2014; Terhorst *et al.*, 2015). Since BMPR1a signaling is involved in LC differentiation *in vitro* and *in vivo* (Yasmin *et al.*, 2013; Capucha *et al.*, 2018), we needed to exclude the fact that the increased inflammation, measured in the context of increased ear swelling, was a collateral

effect of disrupted LC differentiation that would result in lower number of LCs being present in the epidermis. Therefore, we followed before the beginning of the imiquimod treatment (healthy state) and throughout the course of inflammation the number of LCs present in the epidermis of the skin. Before the beginning of the imiquimod treatment, an intact LC network was detected in the skin epidermis. As treatment began and inflammation started, we observed a descent in the number of LCs that reached its peak at day 3. This was followed by a re-population of the epidermis by the LCs, most likely due to the recruitment of bone marrow-derived precursors, will full restoration of the LC network by day 7. Interestingly, no differences were detected between the WT and BMPR1aΔCD11c mice before and during the course of the inflammation, indicating that the observed effect is not simply due to lack of LCs. This supports our claim that the observed effect is a consequence of altered LC/DC function due to impaired BMPR1a signaling and not due to impaired LC/DC differentiation.

We here characterized a fundamental function of inflammation-associated LC-like cells in promoting Treg differentiation and accumulation. In more detail, we described that *in vitro* BMP7 differentiated LCs, which resemble phenotypically LCs present in lesions of psoriatic skin ($CD45^+CD207^+CD206^+CD1c^+TLR2^+$) (Borek *et al.*, 2020), are stronger in promoting $CD4^+CD25^+FoxP3^+$ Treg differentiation from $CD4^+CD45RA^+$ naïve T cells. In addition, this enhanced effect was associated with a decrease in the population of T cells that express IL-22. IL-22 is a cytokine produced by several populations of immune cells including T cells (Dudakov, Hanash and van den Brink, 2015). Th17 and Th22 can produce IL-22 (Eyerich *et al.*, 2009) and DC-derived IL-23 can promote IL-22 secretion by T cells (Dudakov, Hanash and van den Brink, 2015). This might suggest that BMP7-LCs secrete lower concentrations of IL-23 compared to TGF-β1-LCs when in culture with T cells. However, this aspect was not investigated in this study. In addition, studies performed in the Jurkat human T cell line transfected to express FoxP3 described that following stimulation, IL-22 is a target of the transcription factor FoxP3 (Jeron *et al.*, 2012). Moreover, stimulated human $CD4^+CD25^+$ regulatory T cells expressed lower gene levels of IL-22 in comparison to stimulated naïve $CD4^+CD25^-$ T cells (Jeron *et al.*, 2012). This is mediated by the binding of FoxP3 to the promoter region of IL-22 resulting in the repression of its expression (Jeron *et al.*, 2012; Lin *et al.*, 2014). Consequently, the promotion of $FoxP3^+$ Tregs may limit the priming of other T cells fates like $IL\text{-}22^+$ T cells. Further studies will be necessary in order to correctly address this prospect.

Comparable to what was described with BMP7-LCs, the stimulation of GM-CSF, IL-4-differentiated moDCs with BMP7 increased their ability to differentiate $FoxP3^+$ Tregs, through a mechanism which could be reversed with the addition of a soluble BMPR1a inhibitor.

Intriguingly, in the moDC co-cultures, the restriction of BMPR1a signaling caused by the addition of the soluble inhibitor resulted in slightly increased percentages of T cells expressing IL-22. Even though statistical significance was not reached and the percentages of IL-22$^+$ T cells were low, this might suggest a role of BMP signaling in restricting the secretion of IL-22. Future studies could provide valuable data whether BMPR1a signaling has a role in Th22 differentiation and IL-22 expression.

Numerous studies have described that BMPs can skew macrophages towards the anti-inflammatory M2 phenotype which is characterized by the production and secretion of high amounts of the anti-inflammatory cytokine IL-10 (Rocher *et al.*, 2012; Singla, Singla and Wang, 2016; Martínez *et al.*, 2017). We therefore, checked whether BMP signaling could also increase IL-10 production in T cells in our co-culture system. We compared the levels of IL-10 expression between TGF-β1-LC-primed T cells and BMP7-LC-primed T cells. However, we did not detect any differences in the percentages of CD4$^+$ T cells between the two conditions and BMR1a interference had no impact either. This in addition differed from what we previously published, that is that higher concentrations of IL-10 are measurable in the supernatants of TGF-β1-LC T cell co-cultures than of BMP7-LC T cell co-cultures. However, given that by intracellular flow cytometry we specifically measured the percentages of IL-10$^+$ CD4$^+$ T cells, it could be that the IL-10 detected in the supernatants is being produced and secreted by the LCs.

BMP signaling was described previously to regulate maturation and PD-L1 and PD-L2 expression in moDCs (Martínez *et al.*, 2011, 2014). We therefore measured the expression co-stimulatory molecules in moDCs after treatment with BMP7. We observed similar effects as were described by the previous studies. Following treatment with BMP7, moDCs displayed a slightly more mature phenotype however, this was not consistent throughout the experiments and did not reach statistical significance. Interestingly, BMP7 stimulation resulted in strongly increased expression of the co-inhibitory molecules PD-L1 and PD-L2. We observed similar results in LCs only in the case of PD-L2 expression. BMP7-LCs had significantly higher PD-L2 expression in comparison with TGF-β1-LCs but no striking differences were observed in terms of PD-L1 expression (even slightly lower expression in BMP7-LCs compared to TGF-β1-LCs). Regarding the co-stimulatory molecules, we previously published that no strong differences could be detected when comparing TGF-β1-LCs and BMP7-LCs (Borek *et al.*, 2020). These observations could have a strong impact in terms of understanding which molecules can be exploited to modulate DC function, which could be of use in terms of improving DC-based vaccination strategies against tumors or infectious diseases. PD-L1 and PD-L2 expression is often associated with a non-favorable prognosis and worse overall

survival in some cancers (Okadome *et al.*, 2020) and numerous studies have detected that tumor cells can secrete BMPs or that tumor microenvironments are rich in BMPs (Martínez *et al.*, 2017; Valencia *et al.*, 2019; Cortez *et al.*, 2020). Therefore, this might represent a strategy that is used by tumors to promote immune-evasion. Blockade of BMP signaling in the tumor micro-environment could improve protective immunotherapy by blocking the expression of PD-L1 and PD-L2. Studies involving the use of inhibitors of BMP signaling like dorsomorphin, follistatin, or noggin could provide more details regarding their effects and whether this could be proposed as a therapeutic target.

A key finding of our investigation was that BMPs are secreted in BMP7-LC-T cell co-cultures, and that inhibition of BMPR1a activation can retract the increased ability of BMP7-LCs to promote Treg differentiation from naïve $CD4^+$ T cells. By performing a protein array screen for TGF-β family ligands, we were able to detect TGF-β1, BMP2, and BMP7 in the supernatants of the LC-T cell co-cultures. Inhibition of BMPR1a signaling obtained through the addition of a soluble BMPR1a construct to the cultures was able to decrease the greater ability of BMP7-LCs to promote Treg differentiation to levels that were similar to those measured in the TGF-β1-LC-T cell co-culture condition. Similar results were observed also in the case of moDCs. Stimulation of moDCs with BMP7 led to the differentiation of higher percentages of $FoxP3^+$ Tregs which also could be reversed with the addition of an inhibitor of BMPR1a. BMP7 was also detected in the co-cultures between BMP7-moDCs and T cells. In addition, previous studies have reported that following stimulation, moDCs also produce BMP2 and BMP4 (Martínez *et al.*, 2014), which were also described to be able to promote Treg differentiation (Lu *et al.*, 2010). This revealed that in reaction to released agonists, BMPR1a signaling critically participates in the heightened Treg stimulatory capability of BMP7-LCs and BMP7 stimulated moDCs.

We then described that the secreted BMPR1a agonists act directly on the T cells by using *in vitro* models of Treg differentiation in which no APCs are present. In accordance with previous studies (Martínez *et al.*, 2015), we showed that naïve $CD4^+$ T cells possess the machinery to respond to BMP ligands by expressing the gene encoding for BMPR1a. At a protein level, however this receptor is poorly expressed. Following anti-CD38/anti-CD28 stimulation and activation, T cells express quickly higher amounts of the BMPR1a protein. Therefore, following activation T cells can respond to BMPR1a ligands. The addition of BMP7 to anti-CD3-stimulated naïve $CD4^+$ T cells in the presence of IL-2 led to the differentiation of higher percentages of $FoxP3^+$ Tregs. These observations in human cells corroborate and strengthen what was previously shown in mice that lack BMPR1a in the $CD4^+$ T cell compartment. Lower $FoxP3^+$ Treg percentages could be measured in these mice and naïve T

cells isolated from these mice did not efficiently differentiate in FoxP3$^+$ Tregs when compared to WT mice (Kuczma, Kurczewska and Kraj, 2014). In addition, previous studies using murine naïve CD4$^+$ T cells described that other TGF-β family ligands, including BMP2, BMP4 (Hager-Theodorides *et al.*, 2002), and activin A (Huber *et al.*, 2009), can promote Treg differentiation. Additionally, when we treated the stimulated naïve T cells with BMP7, these produced less IFNγ when compared to the vehicle controls. This raises the question whether BMP signaling is also able to inhibit differentiation of naïve CD4$^+$ T cells into Th1 cells or if this is a mere consequence of the fact that higher amounts of FoxP3$^+$ T cells are present in culture and these can block the secretion of IFNγ from the other activated T cells. However, the role of BMP signaling in Th1 response is not so clear. Previous studies performed in murine naïve T cells, that were either deficient for BMPR1a or treated with DM, evidenced that this signaling pathway does not play a role in Th1 differentiation or IFNγ secretion (Yoshioka *et al.*, 2012; Browning *et al.*, 2018). Then again, a more recent paper by Cortez *et al.* demonstrated that BMP7 can negatively regulate IFNγ expression in CD4$^+$ T cells (Cortez *et al.*, 2020). Moreover, we describe that BMP signaling is involved the expression of the IL-2 receptor alpha chain CD25 and that it affects IL-2 production by T cells. These data are in line with previous studies performed in murine cells showing that BMP signaling is involved in activation, homeostasis, and proliferation of T cells (Yoshioka *et al.*, 2012; Martínez *et al.*, 2015).

Our data not only support the fact that BMP receptor signaling promotes human Treg differentiation but also promotes, to our knowledge for the first time, a model in which separate and sequential roles for BMP signaling versus the classical TGF-β1 signaling come about in the generation of FoxP3$^+$ Tregs from naïve CD4$^+$ T cells. TGF-β1 is by far the most well-known cytokine in the context of differentiating FoxP3$^+$ Tregs *in vitro* (Chen *et al.*, 2003; Chen and Ten Dijke, 2016) and is known to not only constitutively signal through the TGFβR1 (i.e. the classical TGF-β1 signaling cascade) but also to signal via a non-constitutive pathway by signaling through BMPR1a/ALK3 (Daly, Randall and Hill, 2008; Keller *et al.*, 2011; Yasmin *et al.*, 2013)

Naïve T cells first acquire high levels of CD25 before gaining FoxP3, resulting in three consecutive differentiation stages (I: CD25$^{-/lo}$; II: CD25$^+$FoxP3$^-$; III: CD25$^+$FoxP3$^+$). Interestingly, inhibition of canonical TGF-β1 signaling (by adding the TGFβR1 inhibitor SB431542) selectively abrogated FoxP3 induction (stage III) resulting in the accumulation of CD25$^+$Foxp3$^-$ cells (stage II). On the other hand, the addition of DM influenced the cell transition from stage I to II, but this effect was not followed with the abrogation of the induction of FoxP3 by the remaining cells. This effect might be explained by the fact that BMP7 triggers the BMP cascade without co-activating TGFβR1/ALK5 signaling, thus promoting he

accumulation of cells in the stage II in the Treg generation cultures. *In vivo*, several TGF-β ligands have been described to be overexpressed and to play an anti-inflammatory role in inflamed tissues (TGF-β1, BMP4, BMP7) (Takabayashi *et al.*, 2014; Kashiwagi *et al.*, 2017). We revealed that simultaneous addition of BMP7 to TGF-β1 additionally promoted Treg differentiation.

The three consecutive stages of Treg generation that were observed in the APC-free system were likewise observed in the co-cultures with LCs. BMP7-LC co-cultures exhibited higher percentages of $CD25^{+}FoxP3^{-}$ cells in comparison to TGF-β1-LC co-cultures. Therefore, observations from two independent Treg generation models validated that BMPR1a signaling induces the promotion of $CD25^{hi}FoxP3^{-}$ cells. Interestingly, inhibition of BMPR1a signaling through the use of a soluble BMPR1a-Fc chimera protein was unsuccessful in inhibiting Treg differentiation TGF-β1-LCs-T cell co-cultures, suggesting that BMPR1a does not play a role in Treg differentiation within these experiments. Among several possibilities, it should be considered that Treg generation in LC co-cultures occurred in the absence of exogenously added TGF-β ligands; therefore, additional factors, including cell contact dependent factors, might also contribute to Treg generation by LCs. Additionally, the added BMPR1a protein might be insufficient to fully block BMPR1a signaling in LC-T cell co-cultures.

We describe for the first time that a portion (30%-40%) of FoxP3 cells in the skin lesions are positive for the expression of the BMP downstream signaling molecule pSMAD1/5/8 in skin biopsies taken from the lesions of psoriasis patients. In contrast, no $FoxP3^{+}$ cells expressed pSMAD1/5/8 in the skin of healthy controls. This supports our notion that during psoriasis there is strong activation of the BMPR1a-dependent cascade and that this is not only limited to keratinocytes and LCs in the epidermis but also extends to $FoxP3^{+}$ cells that accumulate in psoriatic skin lesions.

Our mechanistic studies on the involvement of BMP vs classical TGF-β signaling in Treg generation not only supports a role for BMP signaling in inflammatory Treg accumulation but also provides a model for further studies of inflammation-associated Tregs. Interestingly, several studies previously showed that certain signaling components of the classical TGF-β signaling cascade including TGFβR1, TGFβRII, and pSMAD2/3 are repressed in lesional psoriatic skin versus normal skin (Doi *et al.*, 2003; Jiang *et al.*, 2017), and other studies demonstrated that Tregs from psoriatic patients are functionally defective in their capability to suppress effector T cells (Sugiyama *et al.*, 2005). In support of our findings, BMP2 and BMP4 were also shown to promote Treg generation when added *in vitro* to murine Treg generation cultures (Semitekolou *et al.*, 2009; Lu *et al.*, 2010; Yoshioka *et al.*, 2012; Kuczma, Kurczewska and Kraj, 2014). In addition $FoxP3^{+}$ Treg depletion studies in murine psoriasis-like

inflammation models indicated that FoxP3$^+$ Tregs are functionally active and serve as an important tool in limiting an excessive inflammatory response (Hartwig *et al.*, 2018; Stockenhuber *et al.*, 2018). To our knowledge, our investigation is the first to describe that BMP signaling exerts an important part in inflammatory Treg accumulation during skin inflammation.

Our observation that following stimulation naïve CD4$^+$ T cells upregulate BMPR1a that enables them to respond to BMP ligands in order to favor Treg differentiation by first promoting CD25 expression on naïve CD4$^+$ T cells which then can acquire FoxP3 may be further studied in the setting of induced-Treg generation for the treatment of autoimmune diseases. Today, various studies have generated different established model for the differentiation of FoxP3$^+$ Tregs *in vitro* (Lan *et al.*, 2012). All of these established models rely on the use of TGF-β1 to promote high percentages of Tregs and subsequent studies have observed that concomitant treatment of naïve CD4$^+$ T cells with TGF-β1, IL-2 and other factors including like retinoic acid, rapamycin, butyrate, and others can improve the percentages or number of Tregs that are obtainable (Schmidt *et al.*, 2016). Future studies that will take into account the time kinetics of BMP addition or pre-treatment of naïve T cells with BMPs before TGF-β1 treatment could have a positive impact on the current protocols used for the generation of Tregs by improving the yields. Additionally, our LC-T cell co-culture model system might be applied to naïve T cells isolated from patients suffering from psoriasis or other autoimmune diseases, towards further studying potential T cell intrinsic aberrancies in these patients.

In conclusion, lesional psoriatic skin presents a strong BMP signature and BMP7 expression positively correlates with the accumulated Tregs in the inflamed skin. Our data describe a model in which the accumulation of FoxP3$^+$ Tregs in psoriatic skin lesions is promoted by BMP signaling. We characterized a role for BMPR1a signaling in instructing lesional DCs to gain improved Treg stimulatory capacity and additionally showed that BMP signaling can additionally directly promote T cells to differentiation into Tregs.

6. Bibliography

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